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Metabolism of Non-Conventional Wine Yeasts

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Já faltou mais… E JÁ ESTÁ!

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This thesis was fully performed at both the Microbiology & Biotechnology Group (M&B-BioISI) at TecLabs and at the Enzymology Group (CQB - Centro de Química e Bioquímica) at the Faculdade de Ciências da Universidade de Lisboa, under the direct supervision of Doutora Marta Sousa Silva in the scope of the Master in Applied Microbiology of the Faculdade de Ciências da Universidade de Lisboa.

ABSTRACT

Non-conventional yeast species comprehend a wider physiological diversity that the commonly used *Saccharomyces* yeasts. Their use in wine production goes beyond the production of sensory-active compounds. They have gained interest in the production of wines with increased organoleptic profile and lower alcohol content. This is highly crucial for the wine industry, given the alterations in the grape composition due to climate change. These changes, mainly lower acidity, changes in phenolic and tannin contents, altered maturation, and increased sugar concentration, contribute to increase the alcohol content in wines, modify sensory attributes and wine microbiology. To tackle this problem, it is important to target not only every stage of grape production and winemaking, but also aspects related to microbiological approaches and the selection of non-conventional yeasts for wine fermentation.

The goal of this work was to characterize the metabolism of two selected non-*Saccharomyces* yeasts (*Lachancea thermotolerans* and *Metschnikowia pulcherrima*) grown in a synthetic must matrix under different sugar and nitrogen concentrations. Growth was followed for 71 hours, with sample collection at different timepoints. At the end of cell growth, a DNA fingerprinting analysis confirmed the strain's identity in the culture. Metabolite analysis at different growth stages, in both cells and culture medium, was performed following an untargeted metabolomics approach using Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS). Based on the metabolic profiling, the different strains and culture media could be segregated. Relevant metabolites known to contribute to wine flavour and aroma were selected to uncover specific pathways and metabolic differences between the studied yeast strains in the different growth conditions. These differences found so far as a result of the different media compositions will contribute to the production of novel wines, both in flavour and aroma.

Key-words: Wine, Lachancea thermotolerans, Metschnikowia pulcherrima, metabolism, metabolic profiling

RESUMO

O vinho é um produto com significado cultural e social desde tempos antigos, estando intimamente ligado à história do ser humano e à economia de alguns países. No entanto, a indústria vínica está a ser afetada pelas alterações climáticas, que têm vindo a tornar a produção de vinho conforme os típicos parâmetros comerciais cada vez mais difícil de atingir, enquanto mantendo parâmetros de qualidade segundo a preferência dos consumidores.

Estudos a nível mundial confirmam a tendência de aquecimento a nível global das temperaturas climáticas acima das típicas variações normais experienciadas de ano para ano e que induzem o efeito vintage, pequenas variações na qualidade do vinho e rendimento obtido. A subida constante das temperaturas à escala global modificam o potencial de maturação das vinhas, levando à alteração da composição das uvas que se traduz maioritariamente na modificação do conteúdo em taninos e compostos fenólicos, aumento da maturação e maiores concentrações de açúcares. Maiores subidas de temperaturas apontam para maiores aumentos no teor de açúcares no mosto de uva, que apresenta como principal problema para a indústria vínica maiores teores de álcool nos vinhos após fermentação, assim como uma alteração dos atributos sensoriais e da microbiologia vínicas. Teores de etanol acima dos parâmetros comerciais podem impedir a comercialização dos vinhos produzidos. Os elevados teores de etanol obtidos ao fermentar mostos de elevadas concentrações de açúcares devem-se à elevada capacidade fermentativa e de processamento de açúcares das espécies de leveduras utilizadas como culturas fermentativas, que leva a um elevado rendimento no consumo de açúcar e produção de etanol. Elevadas concentrações de álcool favorecem a ocorrência de fermentações incompletas, levando à decomposição do mosto. Além das altas concentrações de etanol, as elevadas temperaturas podem levar a uma menor acumulação de metabolitos cruciais para o desenvolvimento dos perfis organolépticos vínicos durante o desenvolvimento e crescimento das vinhas. Em adição, a actual utilização generalizada de culturas *starter* para iniciar a fermentação de vinhos na indústria vínica tem conduzido a uma convergência dos perfis sensoriais dos vinhos, que se apresentam cada vez mais constantes e semelhantes entre si, sendo hoje em dia crucial a introdução de maior variedade aromática na maturação dos sabores de vinho para dar continuidade ao desenvolvimento do mercado e à atracção ao consumidor. A utilização das mesmas estirpes de *S. cerevisiae* para iniciação de fermentações tem-se vindo a manter graças às vantagens que o profundo conhecimento da fisiologia, ciclo de vida, metabolismo, processos de regulação e genoma *consensus* destas leveduras confere: o poder de controlar o processo fermentativo sabendo exactamente o que esperar que ocorra durante as diferentes fases do mesmo, assim como um conhecimento prévio do produto que se conseguirá obter, assim como das suas características.

Várias abordagens têm sido feitas para tentar diminuir os níveis de etanol nos vinhos, nomeadamente abordagens viticulturais, pré-fermentativas e pós-fermentativas. Porém, para enfrentar este problema, é importante ter como alvo não só cada etapa da produção das uvas e da produção de vinho, como também as abordagens microbiológicas, de entre as quais uma opção bastante em aberto e inexplorada é avaliar leveduras não-convencionais na fermentação de vinho e selecção de estirpes com potencial. As leveduras não-convencionais compreendem uma maior variedade fisiológica que as leveduras *Saccharomyces*. O seu uso vai além da produção de compostos sensorialmente activos, sendo que têm vindo a ganhar crescente interesse para a produção de vinhos com teores de alcóol mais baixos.

O objectivo deste trabalho foi caracterizar o metabolismo de duas estirpes de leveduras não-*Saccharomyces* seleccionadas, *Lachancea thermotolerans* e *Metschnikowia pulcherrima*. Para tal, ambas as estirpes foram sujeitas a crescimento numa matriz de mosto sintético sob duas diferentes concentrações de açúcares, assim como duas concentrações diferentes de azoto (total de quatro condições cruzadas). As cinéticas de crescimento de ambas as estirpes (em todos os meios de cultura testados) foram avaliadas antes de prosseguir com os estudos. A estirpe de *L. thermotolerans* mostrou um crescimento exponencial típico, tendo-se observado que atingiu taxas de crescimento superiores

quando em meio de mosto sintético com concentrações mais elevadas de açúcar. Já a estirpe de *M. Pulcherrima* mostrou taxas de crescimento substancialmente semelhantes em todos os meios de mosto sintético testados, sendo a taxa de crescimento mais elevada (ainda que muito ligeiramente) em condições de elevado açúcar mas níveis mais baixos de azoto.

Amostras das culturas foram colhidas ao longo do decurso do crescimento para *fingerprinting* das estirpes de forma a confirmar a identidade final das estirpes cultivadas antes de prosseguir para as análises de metaboloma. Foi então conduzida uma análise dos metabolitos em diferentes estágios do crescimento seguindo uma abordagem de *untargeted metabolomics* (análise metabolómica sem alvo específico) utilizando FTICR-MS (espectrometria de massa *Fourier Transform Ion Cyclotron Resonance).* Esta técnica de espectrometria de massa é particularmente relevante no estudo de metabolitos graças à sua elevada sensibilidade, precisão (< 1 ppm) e ultra-elevada resolução (superior a 1,000,000). Normalmente, um estudo metabólico é realizado recorrendo a técnicas como a Ressonância Magnética Nuclear (NMR) e Espectrometria de Massa (MS) ou à combinação destas técnicas a processos de separação cromatográficos como a Cromatografia Gasosa (CG) ou Líquida (LC). Contudo, os limites de deteção por NMR são baixos, e apesar da combinação de MS com CG e LC possibilitar a deteção de um elevado número de compostos, os processos cromatográficos demoram muito tempo e normalmente necessitam de vários passos de limpeza do equipamento. Uma solução será então a utilização de *Fourier Transform Ion Cyclotron Resonance* acoplado a um espectrómetro de massa (FTICR-MS), visto que é um equipamento que não só combina a elevada resolução com a elevada exatidão de massa, como também permite uma rápida e fácil aquisição de resultados.

Foram analisadas amostras recolhidas nos tempos correspondendo ao início da fase exponencial de crescimento e à fase estacionária de crescimento de ambas as estirpes seleccionadas. Após processamento dos espectros em programas de *software* adequado e recorrendo a páginas e ferramentas *online*, foi possível gerar uma lista de compostos com a respectiva distribuição dos mesmos dentro de todas as amostras analisadas. Através de uma análise PCA, foi testada a capacidade de agrupamento através dos perfis metabólicos das diferentes amostras. O agrupamento das amostras deu-se não só por estirpe, como também por tempo e fracção analisada, tendo ocorrido uma boa separação das diferentes amostras.

Os metabolitos relevantes já estudados e que se sabe contribuir para o perfil organoléptico e aroma do vinho foram seleccionados para revelar vias metabólicas específicas e diferenças metabólicas entre as estirpes de leveduras estudadas nas diferentes condições de crescimento testadas. Estas diferenças encontradas como resultado da diferente composição do meio irão contribuir para a produção de novos vinhos no que toca a sabor e aroma. Duas das vias metabólicas mais relevantes encontradas nestas leveduras e que contribuem para o sabor e o aroma dos produtos vínicos foram o metabolismo do ácido alfa-linolénico e o metabolismo de biossíntese de sesquiterpenóides. Vários metabolitos pertencentes a estas duas vias foram identificados e verifica-se que a sua distribuição ao longo das fracções e tempos estudados se altera não só com a estirpe como também com o meio de cultura utilizado (ou seja, com as condições de açúcares e de azoto utilizadas no crescimento das leveduras). Apesar da presença destes compostos em leveduras não-convencionais necessitar de mais estudos, estes microorganismos pouco estudados parecem estar a revelar o seu potencial na indústria vínica. Este trabalho veio validar a criação de perfis metabólicos e a sua utilização para estudos comparativos deste género através da técnica de FTICR-MS aplicada.

Palavras-chave: Vinho, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, metabolismo, perfil metabólico.

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LIST OF ABBREVIATIONS

AES	Air-Exposed Static assays
DAP	Diammonium Phosphate
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediamine Tetraacetic Acid
ESI	Electrospray Ionization
FTICR	Fourier Transform Ion Cyclotron Resonance
FTMS	Fourier Transform Mass Spectrometry
GM	Genetic Modification
HMDB	Human Metabolome Data Base
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LC	Liquid Chromatography
LM	Lipid Maps
m/z	Mass to charge ratio
MS	Mass Spectrometry
NAUC	Net Area Under the Curve
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
rpm	Rotations per minute
SDS	Sodium Dodecyl Sulphate
SM	Synthetic Must
TBE	Tris Borate EDTA
TE	Tris EDTA
Tris	tris(hydroxymethyl)aminomethane
YAN	Yeast Assimilable Nitrogen
YEPG	Yeast Extract Peptone Glucose
YEPGA	Yeast Extract Peptone Glucose Agar

LIST OF EQUATIONS

Chapter 1. INTRODUCTION

Wine has stood as a cultural and social symbol since ancient times, performing a key role in some countries' economy. However, climate changes are taking a toll in winemaking industry, making wine production within commercial and consumer satisfying parameters increasingly difficult (Mozell & Thach, 2015).

1.1. Impact of Climate Change on Wine Production

Vine growth is deeply affected by climate conditions and temperature which strongly influence vine phenology, particularly date of bud break, flowering and *véraison* stages (Fraga et al., 2015; Van Leeuwen & Darriet, 2016).

1.1.1. Climatic Trend and Consequences to Grape composition

Worldwide studies have indicated a clear strong warming trend in climatic temperatures (reviewed in Duchêne & Schneider, 2005; Jones, White, Cooper, & Storchmann, 2005). While year-to-year normal temperature variations induce small variations in yield and quality of wine (the so-called vintage effect), a steady increase of global temperatures shifts maturity potential of grapevines, evidenced as unbalanced ripening profiles (Jones et al., 2005).

Warmer temperatures (as well as higher) translate into earlier sugar ripeness and modifies grape composition (Jones, 2007; Keller, 2010; Mozell & Thach, 2015). The higher the temperature, higher the sugar accumulation (Coombe, 1987). Produced musts will present higher sugar levels than those from grapes grown in ideal conditions. Grape musts with higher sugar contents lead to wines with higher ethanol contents after alcoholic fermentation by currently used yeast species. This is due to their high-fermentative and sugar processing capacity that leads to a high yield on sugar consumption and ethanol production (Godden, Wilkes, & Johnson, 2015; Mira de Orduña, 2010). On the other hand, the higher sugar quantities in must can easily lead to wine spoilage after inducing an osmotic stress response in yeast leading to increase of unwanted fermentation by-products, favouring spoilage as well as stuck/sluggish fermentations to happen (Bisson, 1999; Mira de Orduña, 2010).

1.1.2. Effects upon Wine's Organoleptic Profile

Besides higher alcohol levels, during vine development and growth, metabolism can decrease the accumulation of metabolites responsible for wine's organoleptic profile maturation later during fermentation, during which process wine's acidity will be lost as well, resulting in unbalanced wines (Jones, 2007; Mira de Orduña, 2010; Van Leeuwen & Darriet, 2016). These high-alcohol content wines often contain "off-flavours" that are contrary to consumer preferences, such as modified tannin and phenolic compounds and an increased bitterness perception while decreasing perception of positive sensory aroma compounds (Escudero, Campo, Fariña, Cacho, & Ferreira, 2007; Jones et al., 2005).

Besides having an unbalanced profile when comes to consumers' preferences, marketing parameters can preclude commercialization of such wines. Among the formed by-products is acetic acid, which may exceed the legal limits established for production of wines and delivers bad flavour and, due to lower pH, changes in wine ecology, increasing spoilage and degradation risk (Bisson, 1999; Lagace & Bisson, 1990; Mira de Orduña, 2010).

1.2. Approaches to Lower Ethanol Levels in Wine

Several strategies have been proposed to efficiently reduce alcohol content of wines and abolish any negative changes to organoleptic profiles that are consequence of elevated sugar concentrations (Mozell & Thach, 2015). Approaches to lower ethanol content in wines target the various steps in wine production cycle (Contreras et al., 2014; Varela et al., 2015), namely viticulture, pre-fermentation, fermentation and post-fermentation (Ozturk & Anli, 2014).

Vinicultural approaches try to control the sugar content of grapes through the manipulation of factors like composition and irrigation of soil, and leaf area to crop weight ratio (Kliewer & Dokoozlian, 2005; Ozturk & Anli, 2014). Premature harvest of grapes and adaptation of winemaking practices to unripe grapes is also done (Varela et al., 2015).

Pre-fermentation techniques aim at the reduction of fermentable sugars' content in grape must. This is accomplished by dilution of must, sugar processing with glucose-consuming enzymes like glucose oxidase or sugar removal from grape must; most of these techniques are however expensive (Pickering, 2000).

Post-fermentation approaches are focused on the removal of ethanol from wine after fermentation. Used techniques include evaporation, membrane dialysis and reverse osmosis. Dilution of wine and premature arrest of fermentation are cheaper alternatives, at the cost of quality lost, since ethanol determines aging, stability and organoleptic properties of wine, and flavour dilution, caused by wine dilution (Ozturk & Anli, 2014; Pickering, 2000).

A better strategy to lower ethanol in wine is focused on the fermentation process, usually by targeting wine yeasts.

1.2.1. Fermentation Approaches

Fermentation strategies to lower ethanol tackle the fermentation process and aim at controlling ethanol production. These usually target wine yeasts. Practices include genetic modification (GM) techniques, where yeast genome is manipulated (although the use of GM yeasts in wine production is not allowed) and evolutive techniques that take advantage of natural evolution to manipulate yeasts and generate yeasts with lower ethanol yields while maintaining high wine quality (Contreras et al., 2014; Varela et al., 2015).

Adaptive evolution aims at the change of alcohol production metabolism or inhibition of fermentation through redirecting metabolic flow towards other by-products than ethanol. This has shown to be a difficult challenge since redox and energy homeostasis must be kept as well as wine's sensorial balance, while avoiding accumulation of metabolites with harmful effects on wine quality (Ozturk & Anli, 2014).

A shortcut can be taken with yeast strain selection from natural occurring yeast strains present in grape microbiome, with the advantage of easier use approval. Non-*Saccharomyces* yeast strains are a class of fermentative microorganisms that have been little explored but are now starting to take a place under the spotlight (Suzzi et al., 2016).

1.3. Wine-fermenting Yeasts

Yeast species responsible for wine fermentation can be found naturally as a part of grapes' microbiome (Ciani, Comitini, Mannazzu, & Domizio, 2010; Graham H. Fleet, 2003). These can be divided into two distinct classes: conventional and non-conventional yeasts.

1.3.1. *Saccharomyces* Yeasts (a.k.a. Conventional Yeasts)

Conventional yeasts are most commonly used. These belong to the *Saccharomyces* genus, namely *Saccharomyces cerevisiae*. *S. cerevisiae* strains are generally used as wine starter cultures and are nowadays available as commercial strains (Suzzi et al., 2016). They present unique physiological properties such as a high fermentative capacity that translates oenologically into higher ethanol yields (Piškur, Rozpedowska, Polakova, Merico, & Compagno, 2006; Van Leeuwen & Darriet, 2016). However, this implies an ability to virtually convert all the present sugar into ethanol. Thus, facing the modern ambition to maintain lower ethanol contents in alcoholic beverages, *S. cerevisiae* became a yeast strain with non-desired characteristics (Ciani et al., 2016).

Saccharomyces yeasts metabolize sugars through glycolysis, the cell's central metabolic pathway. The production of fermentation by-products is metabolically linked to ethanol production (Fugelsang & Edwards, 2007).

Both fermentative metabolism and ethanol production are guaranteed in the presence of low oxygen levels by the Crabtree effect. This yeast regulatory system, consisting of the repression of respiration towards the alcoholic fermentation process, is observed in all conventional yeasts, but only in a few non-conventional yeasts (Fugelsang & Edwards, 2007; Heinisch, Rodicio, & Heinisch, 2009; Wilson & Walker, 2010).

The great advantage of working with yeast strains as *S. cerevisiae* is the advanced knowledge of its life cycle, physiology, metabolism, regulation processes and consensus genome (Masneuf-Pomarede, Bely, Marullo, & Albertin, 2016). However, their homogeneity is leading to equally homogeneous flavour profiles of wines, going against the development of novel wines (Van Der Westhuizen, Augustyn, & Pretorius, 2000).

1.3.2. Non-*Saccharomyces* Yeasts (a.k.a. Non-conventional Yeasts)

Non-conventional yeasts comprise a much more diverse population than *Saccharomyces* yeasts. Non-conventional yeasts set an important contribution to the flavour profile of wines and present diverse physiological properties allied to lower ethanol yields and higher production of fermentation by-products (Ciani et al., 2016; Contreras et al., 2014; Romano, Suzzi, Domizio, & Fatichenti, 1997). The main reason relies on a distinct metabolic flow distribution during fermentation (Magyar & Tóth, 2011; Milanovic, Ciani, Oro, & Comitini, 2012).

These yeasts are part of grapes' natural microbiota (G H Fleet, Lafon-Lafourcade, & Ribereau-Gayon, 1984; Suzzi et al., 2016). They are responsible for initiating the fermentative process of grape must, but are overpowered by conventional yeasts after the first two to three days (Ciani et al., 2016; Fugelsang & Edwards, 2007). Examples of these yeasts include members of the genus *Torulaspora*, *Candida*, *Hanseniaspora*, *Lachancea*, and *Metschnikowia* (Ciani et al., 2016).

1.4. Metabolic Diversity in Yeasts

Most metabolic pathways are common to all organisms, from bacteria to humans, following the known principle of metabolic diversity and uniformity which states that metabolic cycles equal or similar exist in different organism (Otterstedt et al., 2004).

The consensus pathway that, until the date, has been found common to all yeast species is the glycolytic pathway. The remaining central carbon metabolism pathways are also identical between different yeast species, suggesting a group homogeneity of yeasts (Rodrigues, Ludovico, & Leão, 2006).

On what comes to other by-product-producing pathways, diversity can be found, as well as in metabolic regulation and nutrient uptake mechanisms. These differ in a very broad range, allowing yeasts to show us how much of a heterogeneous and metabolically complex group they really are (Otterstedt et al., 2004; Rodrigues et al., 2006).

The understanding of non-conventional yeasts' metabolism is essential to select better strains and develop novel approaches to lower the ethanol content in wines at the fermentative process level.

1.5. Objectives of this thesis

The main goal of this thesis was to study the growth and the metabolism of two selected non-*Saccharomyces* yeasts, *Lachancea thermotolerans* and *Metschnikowia pulcherrima*, grown in a synthetic must wine matrix under different conditions.

To fulfil this goal, the following tasks were undertaken:

- Growth analysis of both yeast strains under controlled conditions, with sample collection during growth;
- Analysis of metabolites at different time-points following an untargeted metabolomics approach using FTICR-MS;
- Putative identification of metabolites and uncover of specific metabolic pathways;
- Enlightening key metabolic pathways on each strain.

Chapter 2. MATERIALS AND METHODS

2.1. Microorganisms and Microbiological Methods

2.1.1. Organisms

Yeast strains were selected from the LEVEalliance project (Adl38918) final collection, consisting of 2 yeasts derived from a previous Sograpе Vinhos SA project (Project ICONE QREN-POFC nr. 4586, through a partnership with Proenol company (Project FERMDIF) and M&B-BioISI/FCUL investigation group). Selected yeasts were *Lachancea thermotolerans*, referenced as 483|LCH and *Metschnikowia pulcherrima*, reference 134|MET.

2.1.2. Media

Two different growth media were used: a rich medium for yeast growth containing yeast extract, peptone and glucose (YEPG), and synthetic must (SM), a medium that mimicks grape must.

YEPG (Yeast Extract Peptone Glucose)

YEPG media contained 1% (w/v) yeast extract (Biokar diagnosis), 2% (w/v) peptone (Biokar diagnosis) and 2% (w/v) D-glucose monohydrate (MERCK). All components were dissolved in type II water and the media was autoclaved at 121 ºC for 15 minutes.

YEPGA (YEPGAgar) was prepared in the same way with the addition of 1.5% (w/v) of bacteriological agar (Biokar diagnosis) and autoclaved in the same conditions.

SM (Synthetic Must)

SM media was prepared using stock solutions of components (see table in annexes). Four different SM media were prepared, containing different concentrations of sugar and yeast assimilable nitrogen (YAN), to test growth in four different crossed conditions (**Table 1**). After component addition and total volume completion, SM media were filtered through 0.45 µm nitrocellulose membranes (Millipore, Sigma-Aldrich) in a vacuum bomb filtration system.

Table 1. Information about SM media composition. Sugar and YAN concentrations in each used synthetic must media and its designation along the study.

	S1: 200 g/L of total sugars (glucose:fructose, 1:1)	**S2**: 230 g/L of total sugars (glucose:fructose, 1:1)
N1: YAN× 140 mg/L (0.660 g/L DAP*)	S1N1	S2N1
N2: YAN× 300 mg/L (1.414 g/L DAP*)	S1N2	S2N2

×YAN: Yeast Assimilable Nitrogen.

*DAP: Diammonium Phosphate; chemical formula $(NH_4)_2HPO_4$.

2.1.3. Cultures

Every manipulation of yeast cultures was carried in a Telstar Bio II-A laminar flow chamber to ensure sterility. Cultures in liquid medium were performed in a Thermo Scientific MaxQ4000 Incubator Shaker.

Maintenance of cultures

Original cell cultures were conserved at -80 ºC. A 100 µL loop of frozen cell culture was inoculated in 10 mL of liquid YEPG medium and grown overnight under 160 rpm agitation at 28 ºC to reactivate cells. About 100 µL of reactivated cell cultures were grown in YEPGA medium for two days in a 28 ºC incubator. Plates were conserved at 4 ºC until necessary.

2.1.4. Inoculum Preparation

For each growth cycle, 6 pre-inoculums were prepared (duplicates for 3 biological replicas). Per inoculum, a 10 µL loop of plated culture (conserved at 4 ºC in YEPGA) was inoculated in 10 mL of liquid YEPG medium. The inoculated medium was grown overnight under 160 rpm agitation and 28 ºC. A volume corresponding to initial OD_{600nm} of 0.6 absorbance units in final experimental culture volume was collected and centrifuged for 20 minutes at 4000 rpm. Cells were suspended in 1 mL of synthetic must medium and inoculated in 50 mL of synthetic must in a corked Erlenmeyer.

2.1.5. Static air-exposed with discontinuous shaking growth (AES)

For each media condition, 3 biological replicates assays and a negative control assay were performed. Inoculated SM experimental assays' vessels were kept without agitation in a 24 ºC incubator. Growth was followed for 71 h, with samples collected during 4 days as shown in **Figure 1**.

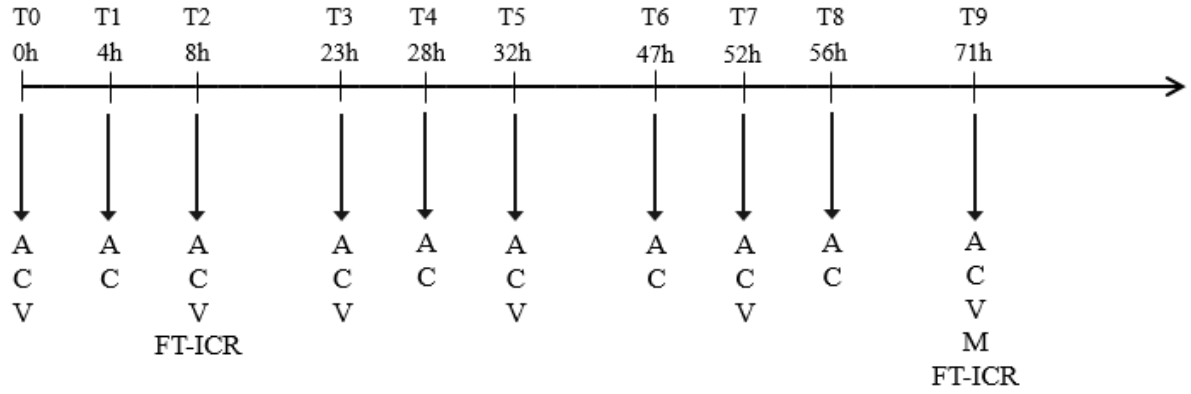


Figure 1. Work flow scheme. Times of sample collecting and techniques applied to each sample, according to the time it was collected. A: Absorbance reading (OD: Optical Density) on 600nm (A_{600nm}); C: Neubauer Cell concentration, using Neubauer's counting chamber (haemocytometer); V: Viable cell concentration determination, by dilution and platting in YEPGA; M: Molecular fingerprinting by PCR (with M13 Primer); FTICR: Metabolite analysis by Fourier Transform Ion Cyclotron Resonance mass spectrometry.

2.1.6. Growth curves (statistical analysis)

Growth curves for each strain were constructed using GraphPad Prism 6.01 software. Statistical analyses were also performed in the same software. Statistical testing to the distribution of data was performed to verify its normal distribution. NAUC values were integrated from growth curves using an automatic EXCEL data sheet provided. Dunnet's test from GraphPad was applied in order to properly compare integrated values of NAUCs. ANOVA analysis were also performed to assesa statistical significance of found differences.

2.2. Analytical methods

2.2.1. Optical density (OD) measurement

Absorbance measurements at 600nm were conducted in Sarstedt Polystyrene 1.6mL Semi-Micro cuvettes (10 x 4 x 45 mm) using a WPA Biotech Photometer UV1101. Calibration of the spectrophotometer was made against negative control SM medium with same composition as used for yeast incubations. Samples were diluted to obtain OD_{600nm} readings between 0.1 and 0.6. Medium used for calibration was also diluted according to the dilution factor of sample preparation.

2.2.2. Cell counts

Cell population counts were carried out in a Hirschmann EM Techcolor haemocytometer (0.100 mm x 0.0025 mm^2) under a Leitz Laborlux K optical microscope. Counts of yeast cells were done across the five diagonal squares. Average cell number per square was calculated following **Equation *1.***.

$$Cells\ per\ \mu L = \frac{Number\ of\ counted\ cells}{Number\ of\ counted\ squares \times {}^{1}/_{400} \times 0.1 \times 10^{-D}}$$, where D stands for used dilution.

Equation 1. Haemocytometer cell counting equation for calculating cell density.

2.2.3. Viable cell counts

Culture samples collected for both studied strains were immediately diluted in 10-fold serial dilutions at collection time. A volume of 100 µL of 10^{-6} and 10^{-8} dilutions were inoculated in YEPGA media using glass beads. Inoculated plates were incubated for 3 days at 28 ºC.

In case of contaminants after growth period, contaminant cultures were streaked out in YEPGA medium and grown in the same conditions. Sampling to observation under optical microscope (Leitz Laborlux K) was also done to assess the nature of contamination.

2.3. Molecular Biology Methods

2.3.1. Genomic DNA extraction from yeast cells

Genomic DNA was extracted from samples collected at the end of growth cycles, following the lithium acetate and SDS method for yeasts for PCR-based applications, adapted from Lõoke, Kristjuhan, & Kristjuhan (2011).

About 100 µL of centrifugated cells were suspended in 200 mM lithium acetate with 1% SDS corresponding to an OD_{600nm} reading of 0.4. The suspension was incubated at 70 ºC in a UBD Grant Thermoblock for 15 minutes. Then, 300 µL of cold ethanol (96-100%, v/v) was added and the suspension was centrifuged for 3 minutes at 15000 g. The pellet was washed using 50 µL of cold ethanol (70%, v/v) and centrifuged for 1 minute at 15000 g. The supernatant was discarded and the tube was left open to evaporate ethanol residues. The pellet was resuspended in 100 µL of TE (10 mM Tris-HCl, pH 8; 1 mM EDTA), frozen 30 minutes at -20 ºC and unfrozen right before PCR reaction. After unfreezing the suspension was centrifuged for 30 seconds and supernatant was collected into a new microtube.

2.3.2. PCR-fingerprinting (Polymerase Chain Reaction)

Strains were analysed by PCR-fingerprinting (with M13 primer) to check for microbiological purity at the end of growth cycles and confirm strains' identity.

Mix for PCR reaction is composed of PCR buffer, nucleotides (dNTPs), magnesium chloride ($MgCl_2$), DNA polymerase, M13 primer (sequence derived from the wild-type phage M13, 5'-GAGGGTGGCGGTTCT-3') and template DNA (extracted according to previous section), as indicated in **Table 2**. The mix was prepared using sterile water. Primers and Taq polymerase were supplied by Invitrogen.

Amplification was performed in a Biometra UNO II PCA DNA thermal cycler, following the program indicated in **Table 3**.

Table 2. PCR composition. Composition of Master Mix prepared for PCR-Fingerprinting with M13 primer.

Master Mix components	Amount used per single reaction (µL)
PCR buffer (10X)	2.5
$MgCl_2$ (50 mM)	1.5
dNTPs (10 mM)	0.5
M13 Primer (50 pmol/µL)	0.5
PCR H_2O	17.8
Taq Polymerase (5 U/µL)	0.2
DNA	2

Table 3. PCR settings. Program settings applied on the thermocycler for PCR-fingerprinting with M13 primer.

Temperature (time)	
95 ºC (5 min)	1 cycle
95 ºC (1 min)	40 cycles
50 ºC (2 min)	
72 ºC (2 min)	
72 ºC (5 min)	1 cycle
4 ºC PAUSE	-

2.3.3. Gel electrophoresis and DNA visualization

A 1.5% (w/v) agarose gel was prepared in 0.5X TBE buffer (Tris-Borate-EDTA, Invitrogen). A volume of 7 µL from each amplification product mixed with loading buffer and 5 µL of 1 kb Plus DNA Ladder (ThermoFisher Scientific, Invitrogen) were applied onto the gel. Electrophoresis ran in 0.5X TBE buffer at 90 V during 1 h and 30 min. Gel staining was performed in a 0.5 µg/mL ethidium bromide solution (Sigma). Imaging was done under an image acquisition system Alliance 4.7 (UVITEC Cambridge).

2.4. Metabolomics

Metabolite profile analysis was performed in supernatant and cell samples corresponding to the beginning and the end of exponential growth phases, in an Apex Qe 7T FTICR-MS (Fourier-Transform Ion Cyclotron mass spectrometer, Brüker Daltonics). Metabolites were analysed through direct infusion with a flow rate of 240µL/h using electrospray ionization in positive mode.

2.4.1. Sample preparation

A volume of 0.5 mL was collected per sample. Collected samples were centrifuged 5 minutes at 15000 g. The supernatant was collected to a microtube and 0.5 mL of 100% methanol (LC-MS grade, Merck) was added. Cells were resuspended in 1 mL of 50% (v/v) methanol, and both cells and supernatants were stored at -20 ºC.

2.4.2. Cell extracts

Cell samples were unfrozen and centrifuged at 15000 g during 5 minutes. The supernatant was discarded and cells were suspended in 1 mL of new 50% methanol solution. Cell samples were then subject to 30 seconds of vortex and 30 seconds in ice. This cycle was repeated 4 times. Samples were once again centrifuged at 15000 g for 5 minutes and the supernatant was collected to a new microtube.

2.4.3. Sample analysis

Before injection, the chosen sample was diluted 1000-fold in a 50:50 solution of methanol:water for analysis by electrospray ionization in positive mode (ESI^+). Supernatant samples

required no additional preparation before MS analysis. A volume of 1 µL of formic acid (Sigma Aldrich, MS grade) was added to potentiate positive ionization of compounds, as well as 0.5 µL of leucine-enkephalin at 1 µg/mL (YGGFL Sigma Aldrich). Leucine-enkephalin was used as an internal standard for quality assessment of analytical precision. For this standard, the molecular mass of [M+H]+ = 556.276575 Th was considered for analysis by ESI^+.

Metabolite analysis was performed by direct infusion in a 7-Tesla Apex Qe Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR-MS, Brüker Daltonics). Mass spectra were acquired by accumulating 100 scans of time-domain transient signals in 512k-point time-domain data sets. Mass accuracy is better than 2 ppm with external calibration and better than 1ppm with internal calibration (using the standard leucine enkephalin). Mass spectra were recorded in the mass range from 100 to 1000 *m/z*. Cleaning of the injection system was performed between samples with acetonitrile (Merck, LC-MS grade).

2.4.4. Data Analysis

Data obtained was analysed in Compass DataAnalysis software V. 4.1 (Brüker Daltonics) to determine the *m/z* values of all obtained peaks. List of *m/z* values was obtained using FTMS parameters, without isotopic deconvolution.

M/z values and corresponding intensities, in a two-column matrix format, were submitted to MassTRIX online platform (Mass Translator into Pathways, masstrix3.helmholtz-muenchen.de/masstrix3/, last accessed in 27th July 2017, Suhre & Schmitt-Kopplin, 2008) to identify possible compounds. Metabolite search was performed in positive ionization mode, considering the possible adducts M+H, M+K and M+Na. Maximum error was set at 2.0 ppm. Compounds were searched in KEGG (Kyoto Encyclopaedia of Genes and Genomes)/HMDB (Human Metabolome Database)/LipidMaps databases without isotopes. Results were exported to Microsoft Excel.

Resulting output lists were checked for the presence of the internal standard peak $[M+H]^+$ = 556.276575 Th. Internal standard was used for quality assessment of analytical precision through determination of relative standard peak deviation. Standard deviation values for internal standard were checked to confirm if standard deviation was inferior or close to 1 ppm.

A JobCompare analysis of all the obtained lists was done in the same MassTRIX platform. This allows the comparison of compounds between the different samples and corresponding intensities. The resulting file was converted into a binary matrix (containing instead of intensity values a 0 for absence and a 1 for presence of the compound) and duplicates were removed, as well as any rows with a zero sum. The resulting matrix was submitted to NTSYSpc programme (Numerical Taxonomy System V. 2.1, Exeter Software) to perform a PCA (Principal Components Analysis).

2.4.5. Metabolic Pathway Analysis

KEGG unique identifiers (KEGG ID's) were extracted from output lists of MassTRIX. Resulting KEGG ID lists (per sample) were submitted into KEGG Pathway Mapping tool (http://www.genome.jp/kegg/pathway.html), against the *Lachancea thermotolerans* (prefix: lth) and *Saccharomyces cerevisiae* (prefix: sce) pathways. Results obtained showed metabolite distribution across metabolic pathways in each sample, number of total metabolites identified and number of identified metabolites per pathway. Search&Color Pathway tool enabled to localize identified compounds on the metabolic map.

Chapter 3. RESULTS AND DISCUSSION

3.1. Effect of sugar and nitrogen concentrations in yeast growth in Synthetic Must

Climate change is leading to higher sugar concentrations in grapes, mainly being glucose and fructose, and as a result higher amounts of sugars to be fermented by winemaking yeasts (Jones et al., 2005). A direct consequence of elevated sugar concentrations, and due to common commercial *Saccharomyces* wine yeasts' high-fermentative capacity, is the production of wines with higher ethanol concentrations (Mozell & Thach, 2015). On the other hand, higher ethanol levels can cause severe stress to the yeast itself and be toxic (Graham H. Fleet, 2003; Hohmann & Mager, 2003). The initial sugar concentration in grape juice can also be a source of stress for yeasts. The high osmotic pressure caused by high sugar levels in must perturbs the normal growth and fermentation processes, being something that can be prone to inducing problems in fermentations of high sugar grape musts (Gibson, Lawrence, Leclaire, Powell, & Smart, 2007). The use of non-conventional wine-making yeasts can contribute to the reduction of ethanol levels and other fermentation by-products.

Studies using wine yeasts require growth conditions close to those in wine fermentation, and hence an accurate composition of culture media must be pondered. Grape juice can be used, although it constitutes an extremely complex chemical matrix, with varying composition according to factors like grape ripeness and climate. Natural grape must or grape juice is also very hard to sterilize due to high sugar content and high nutritional value which accounts for easy contaminations (Viana, Loureiro-Dias, & Prista, 2014). Therefore, chemically defined grape juice media can be used in studies of wine fermentation to ensure results' reproducibility as a viable representation of natural musts (Henschke & Jiranek, 1993). This is of special importance when studies aim to compare, identify and quantify the evolution of chemical compounds along the fermentation process, since it nulls out any of the known changes in grape composition (Haggerty, Taylor, & Jiranek, 2016). In addition, synthetic grape musts have proved to produce results in high agreement to those obtained using natural grape musts fermentation studies (Henschke & Jiranek, 1993; Varela, Torrea, Schmidt, Ancin-Azpilicueta, & Henschke, 2012).

Yeasts require minimal nutritional conditions to grow and perform fermentation. One of them is known as "yeast assimilable nitrogen", or YAN. This includes all nitrogenous compounds that are metabolically available to yeasts as ammonium salts and amino acids. It is estimated that the minimal nitrogen requirement in grape must should be of 140 to 150 mg of nitrogen per litre (N/L), (Fugelsang & Edwards, 2007). YAN levels are highly variable between grape musts, according to factors like climate, grape variety and grape ripeness (Dukes & Butzke, 1998; Fugelsang & Edwards, 2007). Many grape musts have YAN levels below this threshold (Dukes & Butzke, 1998) but this can be corrected through the addition of diammonium phosphate.

In this thesis, the growth kinetics and behaviour of *Metschnikowia pulcherrima* and *Lachancea thermotolerans* yeast species were analysed. These non-conventional wine-making yeasts were grown in different media conditions to perceive the influence of sugar and yeast assimilable nitrogen (YAN) concentrations on yeast growth dynamics. Sugar sources used in the synthetic must (SM) media were glucose and fructose, and diammonium phosphate was the nitrogen source (as described in section **2.1.2.**). SM media was prepared using two different YAN concentrations crossed with two total sugar concentrations to observe any effects of nitrogen or sugar concentration in yeast growth dynamics or fermentation metabolism.

3.1.1. Growth Kinetics and PCR fingerprinting of *Lachancea thermotolerans* and *Metschnikowia pulcherrima*

Pre-inocula of yeast strains were prepared in YEPG medium as described in Methods. An aliquot of culture was centrifuged and transferred to SM media at timepoint T0 to begin the air-exposed static assays with discontinuous shaking assays (AES assays). Assays were made in triplicate with non-inoculated media as negative controls. A series of samples were collected along the 71 h growth time.

The growth dynamics of both yeast strains were determined in four different crossed conditions of nitrogen and total sugar concentration, S1N1, S1N2, S2N1 and S2N2 (according to **Table *1*** in section **2.1.2**.), for 71 hours. At the end of the growth cycles a PCR-fingerprinting was performed using samples from T9 timepoints (corresponding to 71 hours of growth) to verify the cultures' final purity in terms of microbiological strains present. **Figure 2** shows the resulting agarose gel after fingerprinting and electrophoretic separation.

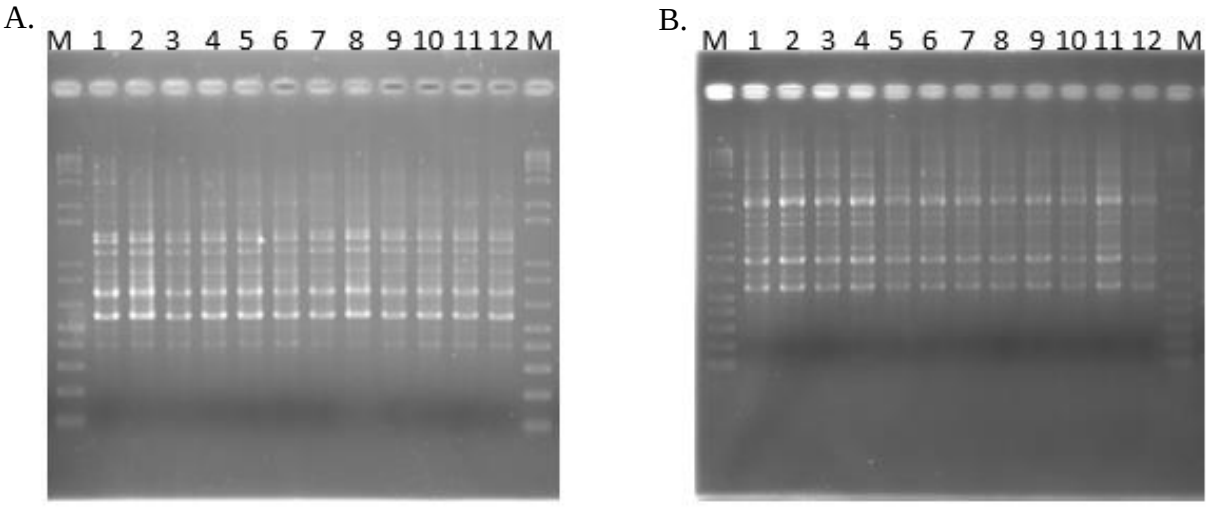


Figure 2. *Lachancea thermotolerans* and *Metschnikowia pulcherrima* PCR profiles amplified with M13 primer. A: Gel corresponding to M13-fingerprinting of *Lachancea thermotolerans*. B: Gel corresponding to M13-fingerprinting of *Metschnikowia pulcherrima*. M refers to molecular ladder (1 kb Plus DNA Ladder supplied by ThermoFisher Scientific, Invitrogen). Lane numbers: 1 to 3, S1N1 replicas 1-3; 4 to 6, S1N2 replicas 1-3; 7 to 9, S2N1 replicas 1-3; 10 to 12, S2N2 replicas 1-3.

The amplification pattern was the same in all samples. Hence, no contaminants were found in any of the samples, meaning the cultures were successfully kept pure until the end of the growth cycles. This allowed for continuation of the experiment proceeding to sample analysis methods.

Cell numbers were determined by measuring the optical density at 600nm. Growth curves of each yeast strain in each SM media were constructed using the mean and error of the triplicates in GraphPad Prism 6.01. The growth curves are shown below (**Figure 3**).

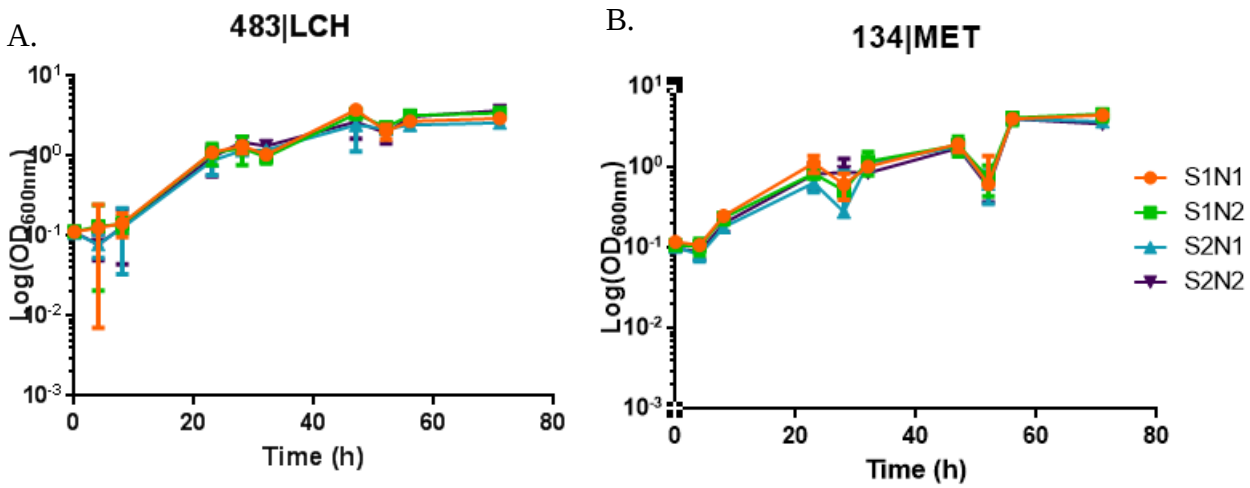


Figure 3. Growth curves of A: *Metschnikowia pulcherrima* (134|MET) and B: *Lachancea thermotolerans* (483|LCH) yeast strains growing in Synthetic Must media with different YAN and total sugar concentrations. Growth curves are represented by the log of the OD_{600nm} versus time (hours). Orange lines represent growth in SM media S1N1, green lines in S1N2, blue lines in S2N1 and purple lines in S2N2. Error bars represent standard deviation from mean of triplicates.

Within each strain, the growth dynamic observed was similar in every growth medium condition. There are not observable differences in terms of growth dynamics within either *L. thermotolerans* or *M. pulcherrima* yeast strains when grown in different YAN and sugar conditions.

In both graphs, the three phases of growth are easily identifiable – a lag phase of adaptation to the media (until 8 hours) is followed by an exponential growth phase (from 8 to 47 hours), ending in a stationary phase (from 47 to 71 hours).

The NAUC (Net Area Under the Curve) of each growth curve was determined using an Excel data sheet. NAUC represents each growth curve through a dimensionless number that accounts for the curve's integral area, which is the area beneath the graph curve. Since the numerical value lacks dimension, a relative quantification of growth, as well as an integrative comparison of growth response between different conditions is allowed. The relation between the NAUCs of the different growth curves can be observed through the radar graphs shown below in **Figure 4**.

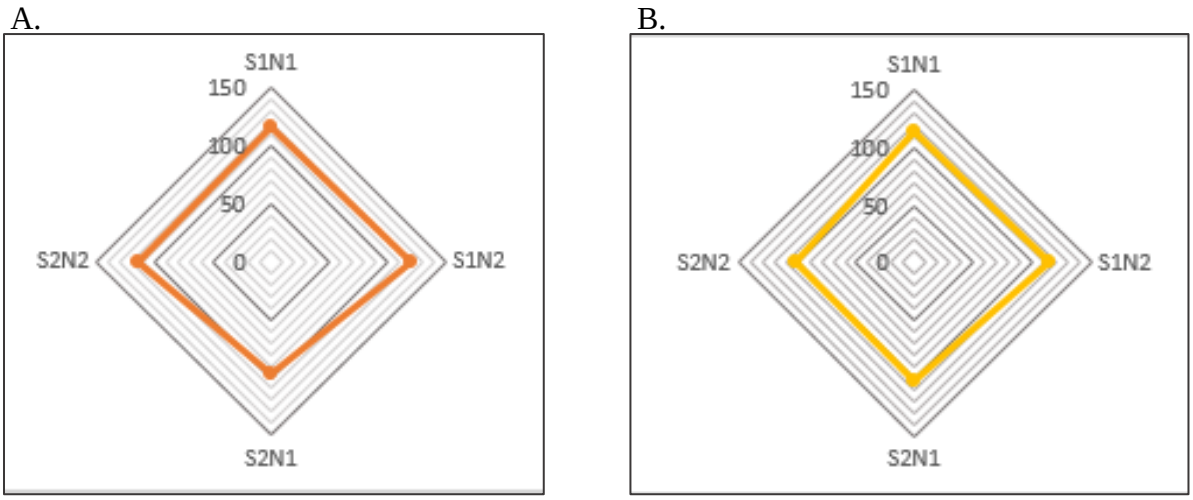


Figure 4. NAUC values for each growth curve, one per different growth media, within each yeast strain. A: NAUC's of *Lachancea thermotolerans* (483|LCH) strain are plotted. B: NAUC's belonging to the *Metschnikowia pulcherrima* (134|MET) strain. Orange line corresponds to *L. thermotolerans*; yellow line corresponds to *M. pulcherrima*.

The analysis of the NAUC values for *L. thermotolerans* and *M. pulcherrima* yeast strains confirmed that the variation within each strain in the various growth media is very small. Between the both studied strains the differences are very small as well.

A two-way analysis of variance (ANOVA analysis) was performed for all growth curves, using NAUC's calculated values for each of the growth curves to evaluate yeasts' growth response changes facing variations in sugar and YAN concentrations. Dunnett's test was performed to compare NAUC's and assess the effect of the different growth conditions. Statistical data is not shown; statistical complementary results can be found in the annexes. The performed analysis showed no significant differences in growth for each strain among the different SM media and pointed that variance between the growth curves is essentially due to sampling errors, and not due to variations between the populations subject to different conditions. This means that there are not any statistically significant differences between the populations subject to different conditions due to the differences between media composition.

Growth rates for *L. thermotolerans* and *M. pulcherrima* yeast strains grown in the different SM media were determined through a linear regression of exponential phase optical density (logarithmic values of OD_{600nm}). For best comparison of growth rates within and between strains, radar charts were constructed and are shown below (**Figure 5**).

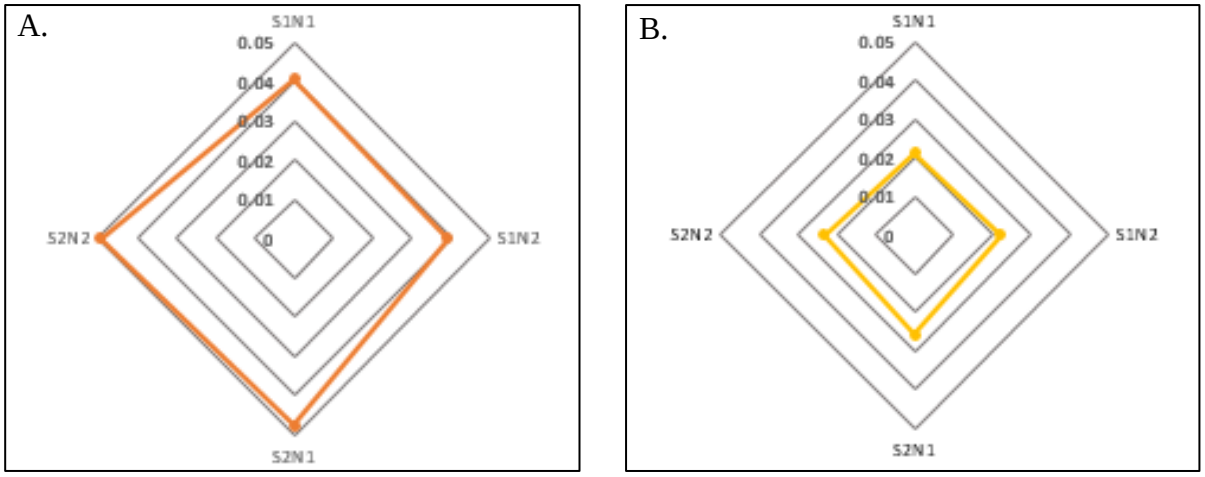


Figure 5. Growth rates of *Lachancea thermotolerans* and *Metschnikowia pulcherrima* grown in different media and its variation graphically represented. Growth rates linearly determined equations can be consulted in the annexes, as well as the percentual growth rate changes. A: Growth rates of *Lachancea thermotolerans* strain. B: Growth rates of *Metschnikowia pulcherrima* strain. Orange line corresponds to *L. thermotolerans* grown in different media; yellow line corresponds to *M. pulcherrima* grown in different media.

Variations of growth rates of *L. thermotolerans* strain (483|LCH) occur when the composition of growth media in terms of global sugars and YAN are shifted. Considering the growth in S1N1 media as standard for relative reference (lowest sugar and YAN), growth rate is approximately the same (lowers only 3.7%) when, in the same sugar condition (S1, 200 g/L), a higher YAN concentration is provided (from 0.041 to 0.039 h^{-1}, see **Tables 6 and 8** in annexes). However, when higher sugar concentration is available (S2, 230 g/L), a global raise of growth rate is observed. For the highest sugar and YAN concentrations tested (media identified as S2N2, 230 g/L of total sugars and 300 mg/L of

YAN), the growth rate of *L. thermotolerans* strain increases by 21.2% (from 0.041 to 0.049 h^{-1}, see **Tables 6 and 8** in annexes).

Change of growth media sugar and YAN conditions led to changes in growth rates of *M. pulcherrima* strain as well (134|MET). The growth rate in the S1N1 media was as well considered a standard for relative reference for analysis. With the *M. pulcherrima* strain, every change in media (in terms of sugar concentration and/or YAN) led to a raise of the growth rate. The highest growth rate (0.026 h^{-1}, 20.7% higher than in standard S1N1 media, see **Tables 7 and 8** in annexes) was observed in the media identified as S2N1 (230 g/L of total sugars and 140 mg/L of YAN). Unlike what happens with *L. thermotolerans* growth, a higher YAN associated with higher sugar concentration does not stimulate faster growth for *M. pulcherrima*, giving only 8.5% increase of growth rate relatively to the standard growth considered (from 0.021 to 0.023 h^{-1}, see **Tables 7 and 8** in annexes).

Comparing both strains, *L. thermotolerans* presents a 2-fold higher growth rate than *M. pulcherrima* in all media analysed. Since the studied strains are from distinct species, they are expected to grow at different rates and respond differently to changes of growth media composition, since, most likely, they have different metabolism.

3.2. Study of yeast metabolome using Fourier Transform Ion Cyclotron Resonance mass spectrometry (FTICR-MS)

Metabolomics is the study of all the chemical compounds present in a cell at a certain moment that result from cellular processes as a last answer of a biological system to any given environmental or genetic changes - the cell's metabolome (Han et al., 2008). Metabolites are a direct reflection of the cell metabolism. Since metabolites tell the story of what happened in the cell, the study of their absence/presence, as well as their relative concentrations within the cell, is the most promising way of phenotypic studies of organisms (Johnson, Ivanisevic, & Siuzdak, 2016).

There is a vast array of methods used for metabolomics studies, being NMR (Nuclear Magnetic Resonance) spectroscopy one the most commonly used techniques one can found. However, when it comes to resolution and mass precision, no method can be better for metabolomics studies than Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometry, with an ultra-high resolution (superior to 1,000,000) and mass precision under 1ppm (Han et al., 2008).

The metabolites of *Lachancea thermotolerans* and *Metschnikowia pulcherrima* yeast strains were analysed in the beginning of exponential growth phase and in stationary phase (end of exponential growth), using Fourier Transform Ion Cyclotron Resonance mass spectrometry (FTICR-MS). This technology allows the detection of molecules with ultra-high resolution (above 1,000,000) and ultra-high mass accuracy (under 1 ppm) (Han et al., 2008).The main objective was to profile the compounds inside cells and compounds secreted to the media surroundings during alcoholic fermentation by both yeast strains, facing different synthetic grape must conditions.

During yeast growth, cells and culture media were collected, according to **Figure 1** (see section **2.1.5.**). To metabolically characterize yeast cells in the different growth conditions tested, samples were collected along the time-course of growth assays. The timepoints T2 (8 h) and T9 (71 h) were chosen since they correspond to the beginning of the exponential growth phase and the stationary phase, respectively.

Samples from growth media and yeast cells from selected timepoints were extracted with methanol. Extracted metabolites were analysed by direct infusion mass spectrometry, in an FTICR mass spectrometer, following electrospray ionization (ESI) in positive ion mode. The number of found ions (peaks) per spectre ranged between 737 and 1952. A representative mass spectrum of *L. thermotolerans* and *M. pulcherrima* in stationary phase is shown in **Figure 6**.

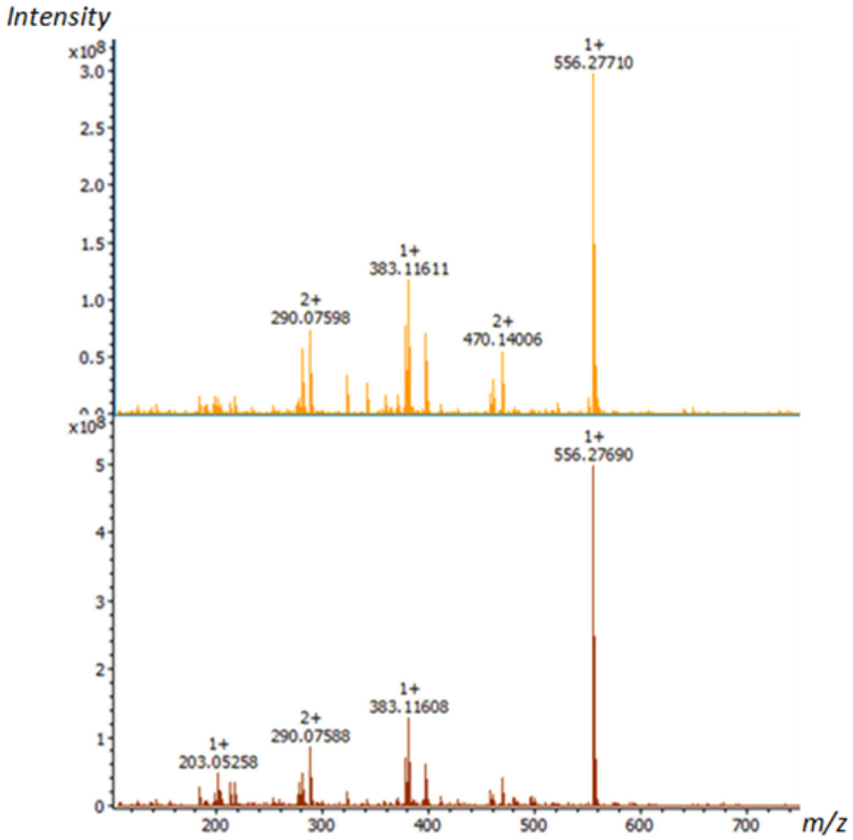


Figure 6. Mass spectra for both strains analysed by direct infusion FTICR-MS operating in positive electrospray mode (ESI^+). Yellow spectrum corresponds to a pool of media sampled from *M. pulcherrima*'s culture and orange spectrum corresponds to a pool of media sampled from *L. thermotolerans*' culture; both spectra are from samples grown under S1N1 conditions and at T9 timepoint (end of growth cycles). The highest peak observed at 556.277 *m/z* corresponds to the standard leucine enkephaline, $[M+H]^+$.

3.2.1. Metabolome analysis

Using the DataAnalysis program by Brüker, a list of all *m/z* values present in each spectre (each one corresponding to a distinct peak) was generated. The resulting lists were filed in an Excel Worksheet with adequate identifications. Each *m/z* list (corresponding to only one sample) was submitted to MassTRIX to identify putative compounds. Search options selected included positive ionization scan mode, considering Na^+ and K^+ as possible adducts, and a maximum error of 2.0ppm.

Each Job ID (reference number attributed automatically to every submission to the MassTRIX Run Tool) was saved to perform a JobCompare in MassTRIX. The resulted Excel file contains all samples represented side by side, and every putative compound identified with KEGG name, KEGG mass, chemical formula, raw mass, peak intensity and deviation in ppm. A total of 8943 different possible compounds were identified, distributed across all samples. This difference in the number of peaks obtained and the number of likely different compounds is because the same mass can be attributed to more than one compound.

To compare the metabolome of both studied strains, a Principal Component Analysis (PCA) was performed using the JobCompare file in the NTSYSpc 2.21q software pack. This statistical exploratory data analysis requires for the data to follow a normal distribution and is based in a mathematical transformation of data that uses orthogonal transformation to convert a group of possibly correlated variables into a group of linearly non-correlated variables, called principal components. Each principal component is not correlated with the previous components. PCA allows seeing if there is any grouping between the samples based on the data obtained.

A matrix containing all data of samples and respective compounds was built in Microsoft Excel; this matrix was afterwards exported to the NTedit 2.21 software. The PCA was performed in NTSYSpc 2.21q.

Projections in 2D and 3D were the resulting outputs, showing the distribution of each sample in chosen dimensions (the dimensions being PC1, PC2 and PC3). The 3D projection can be found in the annexes.

Main results concerning grouping in PCA projections are shown in the figures below. A table to identify the samples by their respective number is also shown (**Table 4**).

Table 4. Identification of all samples shown in PCA analysis figures. Fraction S is supernatant (culture medium) and C corresponds to cells.

Sample number	Growth Media	Strain	Fraction	Timepoint
1	S1N1	CONTROL	-	-
2		483|LCH	S	T2
3				T9
4			C	T2
5				T9
6		134|MET	S	T2
7				T9
8			C	T2
9				T9
10	S1N2	CONTROL	-	-
11		483|LCH	S	T2
12				T9
13			C	T2
14				T9
15		134|MET	S	T2
16				T9
17			C	T2
18				T9
19	S2N1	CONTROL	-	-
20		483|LCH	S	T2
21				T9
22			C	T2
23				T9
24		134|MET	S	T2
25				T9
26			C	T2
27				T9
28	S2N2	CONTROL	-	-
29		483|LCH	S	T2
30				T9
31			C	T2
32				T9
33		134|MET	S	T2
34				T9
35			C	T2
36				T9

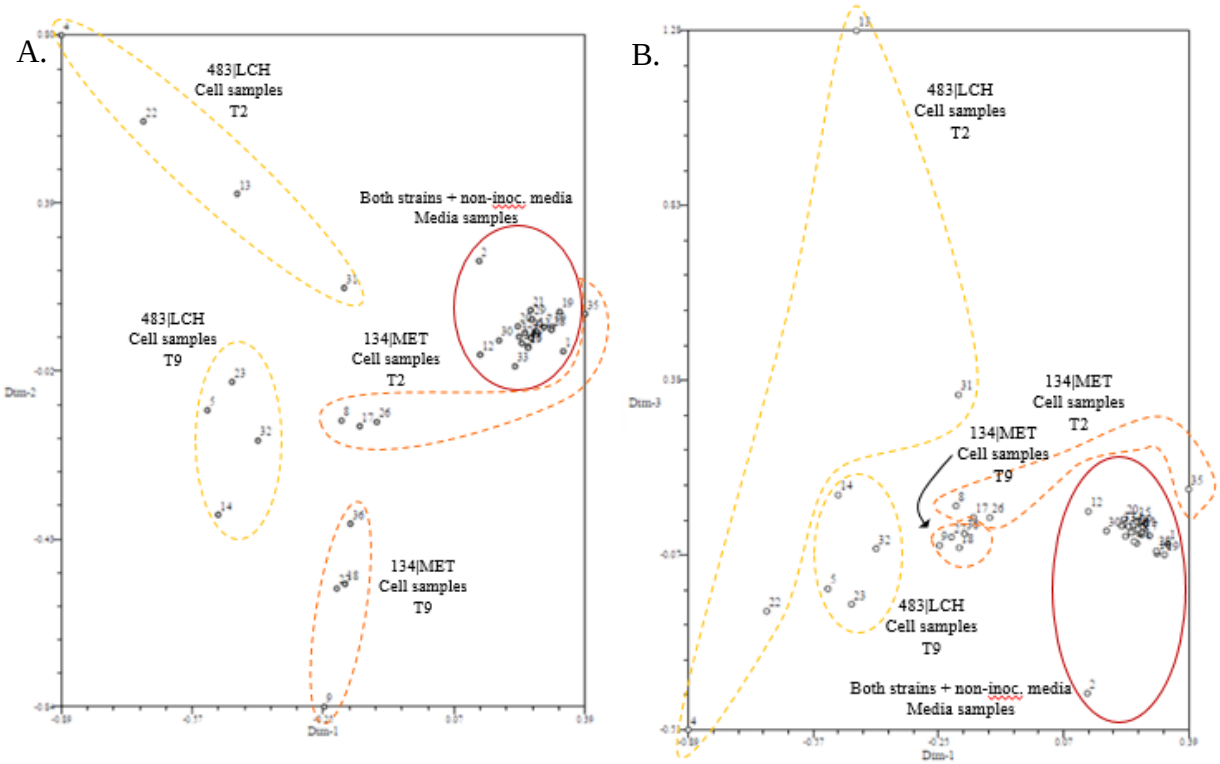


Figure 7. 1st PCA main results from bidimensional projections. PCA was performed using the totality of data. Samples are circled to form sample groups surrounded by the visible lines. Yellow open lines are cell samples of *L. thermotolerans*; orange open lines are cell samples of *M. pulcherrima*; red lines are supernatant samples from both studied strains and non-inoculated media samples. Labels are next to each group. A: Dimensions 1 (horizontal axis) and 2 (vertical axis). B: Dimensions 1 (horizontal axis) and 3 (vertical axis).

There is a clear separation between media supernatant and cell extracts for both yeast strains analysed. There is a clear grouping as well between supernatant samples and non-inoculated medium samples (negative controls).

Within the cell samples, there is a separation between the *L. thermotolerans* (yellow groups) and the *M. pulcherrima* (orange groups) strains. Among each strain, both analysed time-points were present separated.

Two samples didn't fall in any established group, standing as possible outliers in the data: points 35 and 13.

To elucidate if the observed deviation could be due to sample variance or sampling error, a second PCA analysis was performed. For this second analysis, a copy of the previously submitted compound list was made and every single compound present in the non-inoculated media (control samples) were eliminated from every sample, as well as the control samples. Full projections can be found in the Annexes.

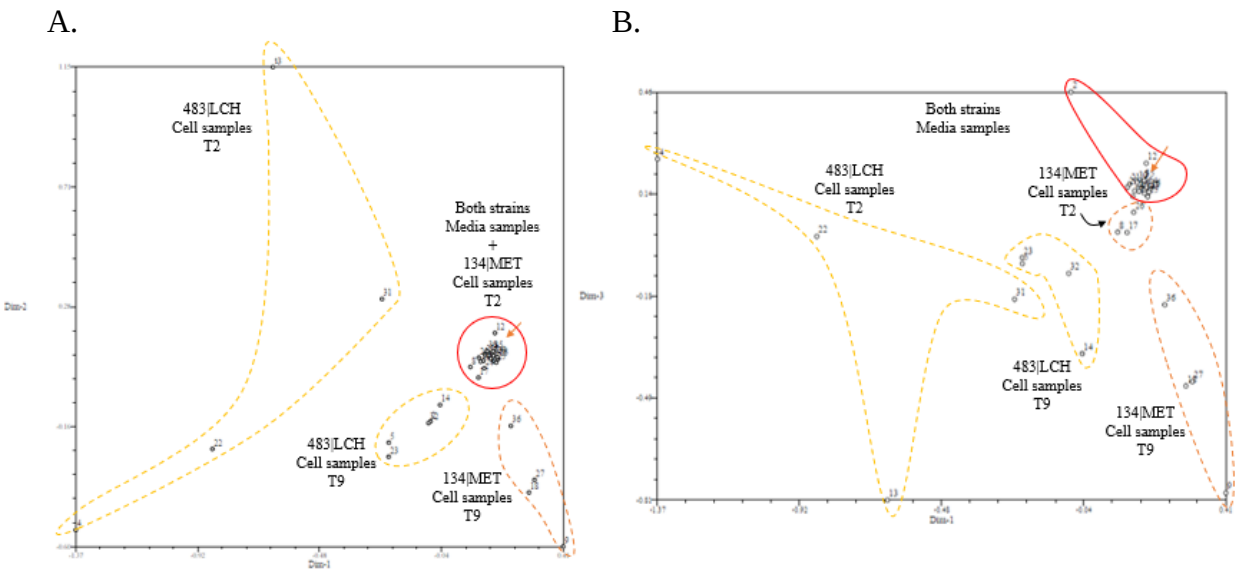


Figure 8. 2nd PCA main results from bidimensional projections. PCA was performed without data belonging to non-inoculated media samples. Samples are circled to form sample groups surrounded by the visible lines. Yellow open lines are cell samples of *L. thermotolerans*; orange open lines are cell samples of *M. pulcherrima*; red lines are supernatant samples from both studied strains and non-inoculated media samples. Orange arrows indicate the position of a point belonging to the orange open lines group in Figure 7.A. and the position of a point belonging to the upper orange open line group in Figure 7.B. Labels are next to each group. A: Dimensions 1 (horizontal axis) and 2 (vertical axis). B: Dimensions 1 (horizontal axis) and 3 (vertical axis).

After this new analysis, we observed that, while point 35's deviation is due to a higher intrasample variability, the point 13 must be a result of true sampling or analysis error, since it appears even further away from what is would be expected.

No new groupings were found in the second PCA, so due to inexistence of any new conclusions the analysis continued using only data from the first PC analysis.

A visual colour pattern was constructed assigning each compound a distinct colour corresponding to the prevalent PC which explained its presence. The compounds explained by the first three principal components made up about 30% of the total compounds found; these were selected and copied into an independent list. Compounds were organized first by PC (from PC1 to PC3) and, within each PC, from the highest absolute PC value to the lowest. The distribution of each compound through all the samples was then analysed; a colour pattern was constructed signalling the presence of each compound across all samples, with a distinct colour attributed to the 3 different PC's. Negative and positive PC values were as well discriminated.

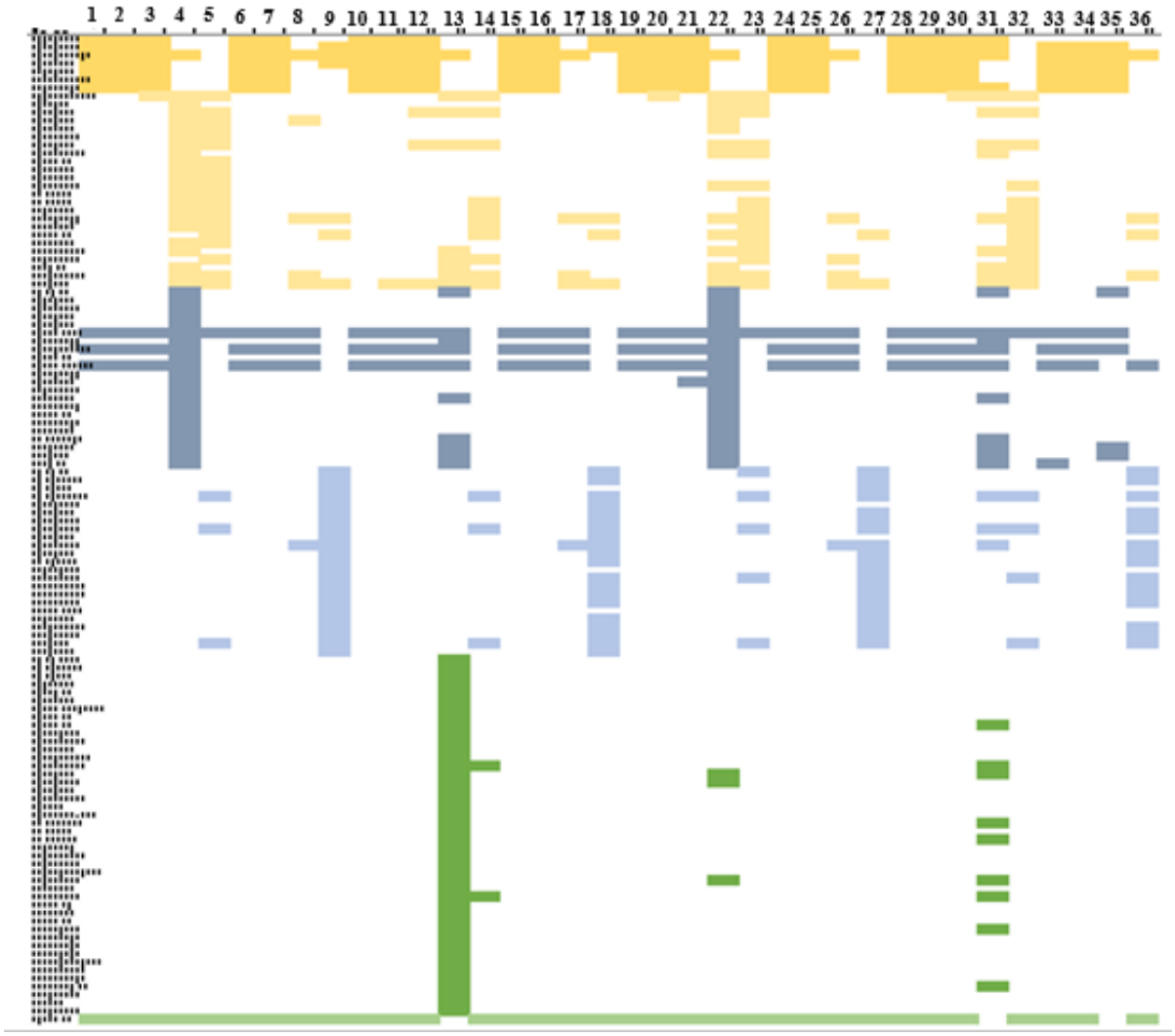


Figure 9. Colour pattern constructed from the PCA results. Each column corresponds to a different sample (detailed in Table 4) and each line corresponds to a different chemical formula. Compounds corresponding to those chemical formulas were separately listed for posterior analysis. Colours establish the found chemical formulas in each sample and colours illustrate which Principal Component (PC) best explained such chemical formula's distribution across samples. Darker colours correspond to positive PC's and lighter colours to negative C's. Yellow illustrates the 1st PC, Blue the 2nd PC and green the 3rd PC.

From this list, with the objective of diminishing the amount of information, a crossover was made with the compounds deemed significant in the second PCA following the concept that compounds that popped up in both PCA's would be more certainly significant.

About 100 compounds in common were found between the first three principal components of both performed analysis. These compounds were deemed as significant since they showed up in both performed PCA's. A list including all these compounds in terms of chemical formulas and their corresponding possible identifications was comprised; the role of each possible identification was searched. The reduced compound pattern is shown below. The extensive list of compounds and their corresponding functions and/or names can be found in the Annexes (**Table *12***).

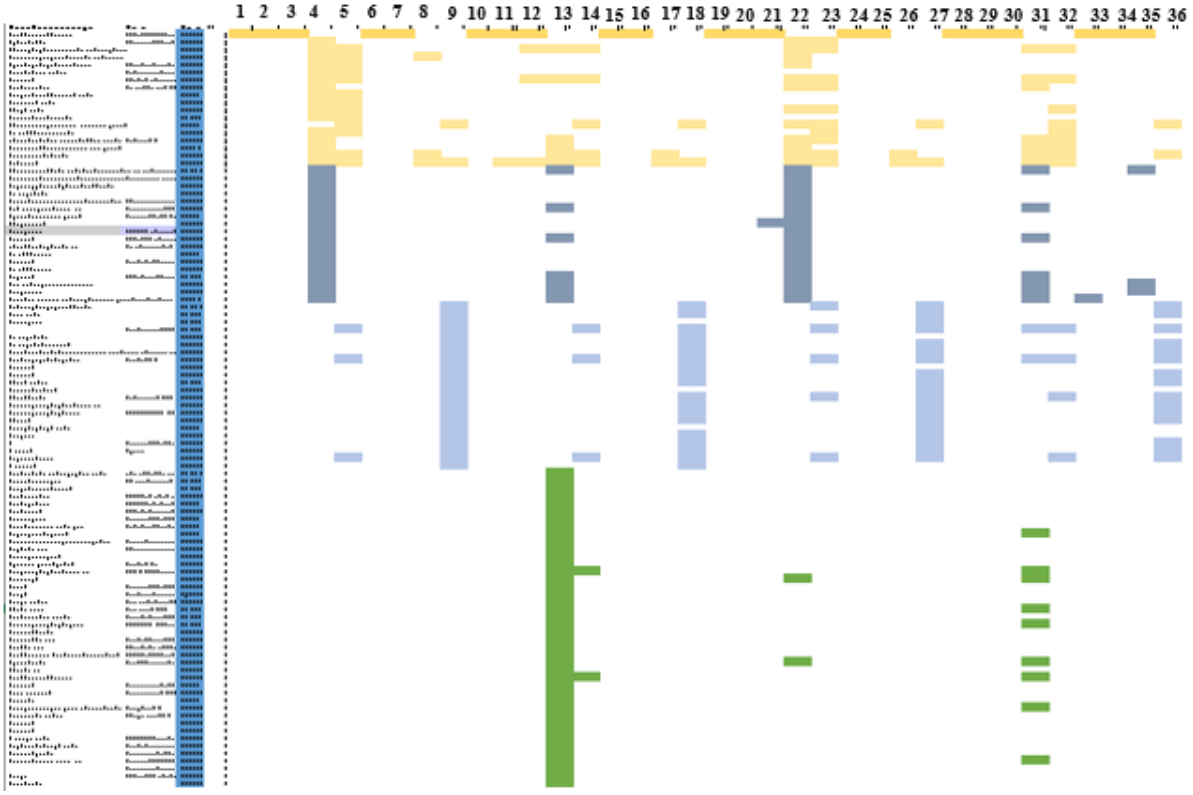


Figure 10. Reduced color pattern from PCA results. Pattern with left compounds after final filtering of data. Each column corresponds to a different sample (detailed in Table 4) and each line corresponds to a different chemical formula. Compounds corresponding to those chemical formulas were separately listed for posterior analysis. Colours establish the found chemical formulas in each sample and colours illustrate which Principal Component (PC) best explained such chemical formula's distribution across samples. Darker colours correspond to positive PC's and lighter colours to negative C's. Yellow illustrates the 1st PC, Blue the 2nd PC and green the 3rd PC.

3.2.2. Metabolite identification and pathway analysis

The major subclasses of compounds found in this final list, representing up 40% of the total compounds, was those of flavonoids, carboxylic acids and other known sensory active compounds typically found in wine. A relatively predominant class of compounds included lipids: fatty acids, linoleic acid metabolism intermediates and arachidonic acid, and D3 vitamin intermediates (in Annexes). About 62% of the sensory active compounds listed in **Table 12** were found in *L. thermotolerans*, but only about 27% of these (about 17% of the total compound list) were present at the end of growth (timepoint T9). Although only about 24% of the total listed compounds (see **Table 12** in annexes) were found in *M. pulcherrima*'s samples, all of them were present at the end of growth cycle (timepoint T9).

The problem of the increased sugar concentrations in grapes and its effect in wine organoleptic profiles, namely the undesired consequence of high ethanol levels after yeast fermentation of grape musts, targeted the metabolome analysis to the effects of glucose variation on *L. thermotolerans* and *M. pulcherrima* metabolic profiles, in both cells and culture media, with discussion of its possible effects upon wine profiles.

Based on the putative identified compounds in both yeast strains, several key metabolic pathways were identified. First, a list of all possible KEGG ID's identified from every single sample was generated. Others that contained only HMDB (Human Metabolome DataBase) ID's were converted to KEGG ID's, leaving ID's from LM (Lipid Maps database) unconverted. Using the online tool "Search&Color Pathway" from KEGG the list of attributed KEGG ID's was submitted to identify the metabolic pathways for each entry code, using *Lachancea thermotolerans* and *Saccharomyces cerevisiae* pathways. Metabolic pathways in common between samples were chosen and the differential presence and absence of found metabolites was evaluated and highlighted.

Two of the most relevant pathways in yeast that contribute to the flavour and aroma in wine are the alpha-linolenic acid metabolism and to the sesquiterpenoid biosynthesis metabolism. We identified several putative compounds belonging to these pathways, that change between strains and the culture media.

Linolenic acid is an essential polyunsaturated fatty acid critical for the stability of yeast cells' membrane, influencing its growth. It is as well a necessary substrate for the formation of C_6 alcohols and aldehydes, which in turn influence wine's organoleptic profile (Waterhouse, Sacks, & Jeffery, 2016). In *Lachancea thermotolerans*, there are significant differences in the alpha-linolenic acid metabolism pathway when cells grow under the same lower YAN levels (N1) with different glucose concentrations (**Figure 11**). With less sugar in the culture medium (S1 condition), metabolism is shifted to the production of the fatty acyls hydroxyoctadecatrienoic acid (2(R)-HOTrE) and heptadecatrienoic acid. Both hydroxyoctadecatrienoic and heptadecatrienoic acids are by-products of yeast metabolism commonly produced in all alcoholic fermentations. The presence of fatty acyls is crucial for biosynthesis of esthers, alcohols and aldehydes that will contribute for impact wine's organoleptic profile (Waterhouse, Sacks, & Jeffery, 2016). For higher YAN concentration (N2), this is not observed, and this metabolic pathway seems identical in detected metabolites in both S1 and S2 conditions.

The strain *M. pulcherrima* does not present significant changes in these metabolites for lower sugar concentrations (S1), but when in presence of higher sugar and YAN (S2N2 medium), it secretes heptadecatrienal and traumatic acid to the culture medium (**Figure 14**). The first metabolite is an aldehyde produced by plants, algae and fungus, with a possible function as a membrane-permeable non-volatile defence compound in plants (Sukatar, Karabay-Yavaşsoglu, Ozdemir, & Horzum, 2006; Weichert, Stenzel, Berndt, Wasternack, & Feussner, 1999). Traumatic acid is a fatty acid derivative, mainly found in plants and algae, acting in the stimulation of cell division and growth and possessing an antioxidant activity (Pietryczuk & Czerpak, 2011, 2012). This strain presents the highest growth rate in S2N1 medium, and in this condition, traumatic acid is produced and remains inside cells (**Figure 13**).

With higher sugar in the medium (and lower YAN, S2N1), both *L. thermotolerans* and *M. pulcherrima* produce and secrete isomethyl-jasmonate and methyl-jasmonate, both volatile derivatives of jasmonate. Jasmonate and its derivatives belong to a signalling pathway for defence responses found in the plant kingdom. Although widely spread in plants, it is still a compound with little understood functions, particularly in yeasts. Jasmonic acid, its precursor, has been found in fungal plant pathogens

(Häusler and Münch, 1997). Methyl-jasmonate belongs to monoterpenoids subclass and has an aromatic typical odour. It has been connected to isoflavonoid metabolism, sensory active compounds commonly found in wine that contribute to its flavour profile (Mander & Liu, 2010; Portu, López, Baroja, Santamaría, & Garde-Cerdán, 2016).

Volatile isoprenoids are aroma compounds that provide wine its character and are responsible for aroma and flavour perceptions in wine (Aslankoohi et al., 2016; Graham H. Fleet, 2003; Institute of Chemistry, 2008). Sesquiterpenoids belong to this wide range of chemical compounds, corresponding to C_{15} compounds. Though many isoprenoids already exist in grapes, since they are common compounds in plants, they are as well produced by yeast cells during wine fermentation (Waterhouse et al., 2016). Several differences have been observed in *L. thermotolerans* and *M. pulcherrima* concerning the production and secretion of sesquiterpenoids upon growth in the different media (**Figures 15** to **18**). One example is the sesquiterpene humulene, secreted by *Lachancea thermotolerans* in S1N2 medium and by as well as *Metschnikowia pulcherrima* in both S2N1 and S2N2.

Although the presence of these compounds in these non-conventional yeast strains requires further studies, the differences in the synthesis of these compounds as a result of the different media compositions may contribute to the production of new wines with different flavours and aroma.

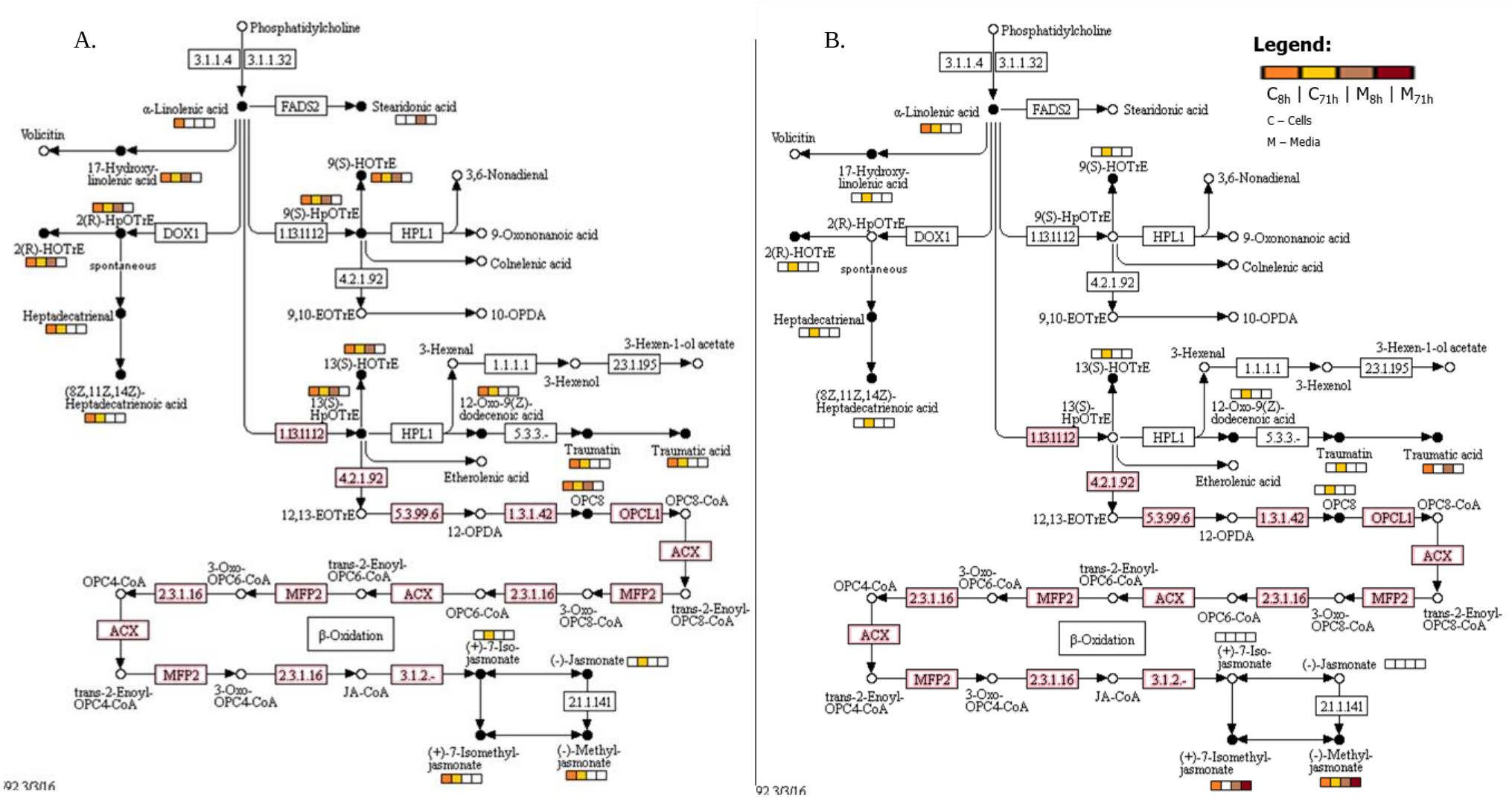


Figure 11. Alpha-linolenic acid metabolism in *Lachancea thermotolerans* in two different sugar conditions. A: corresponds to S1N1 growth; B: corresponds to S2N1 growth. Putatively identified compounds are marked as black full dots. Each square corresponds to a different sample, cells (C) and medium (S) at timepoints T2 (8h) and T9 (71h), (legend included in figure). Image adapted from KEGG.

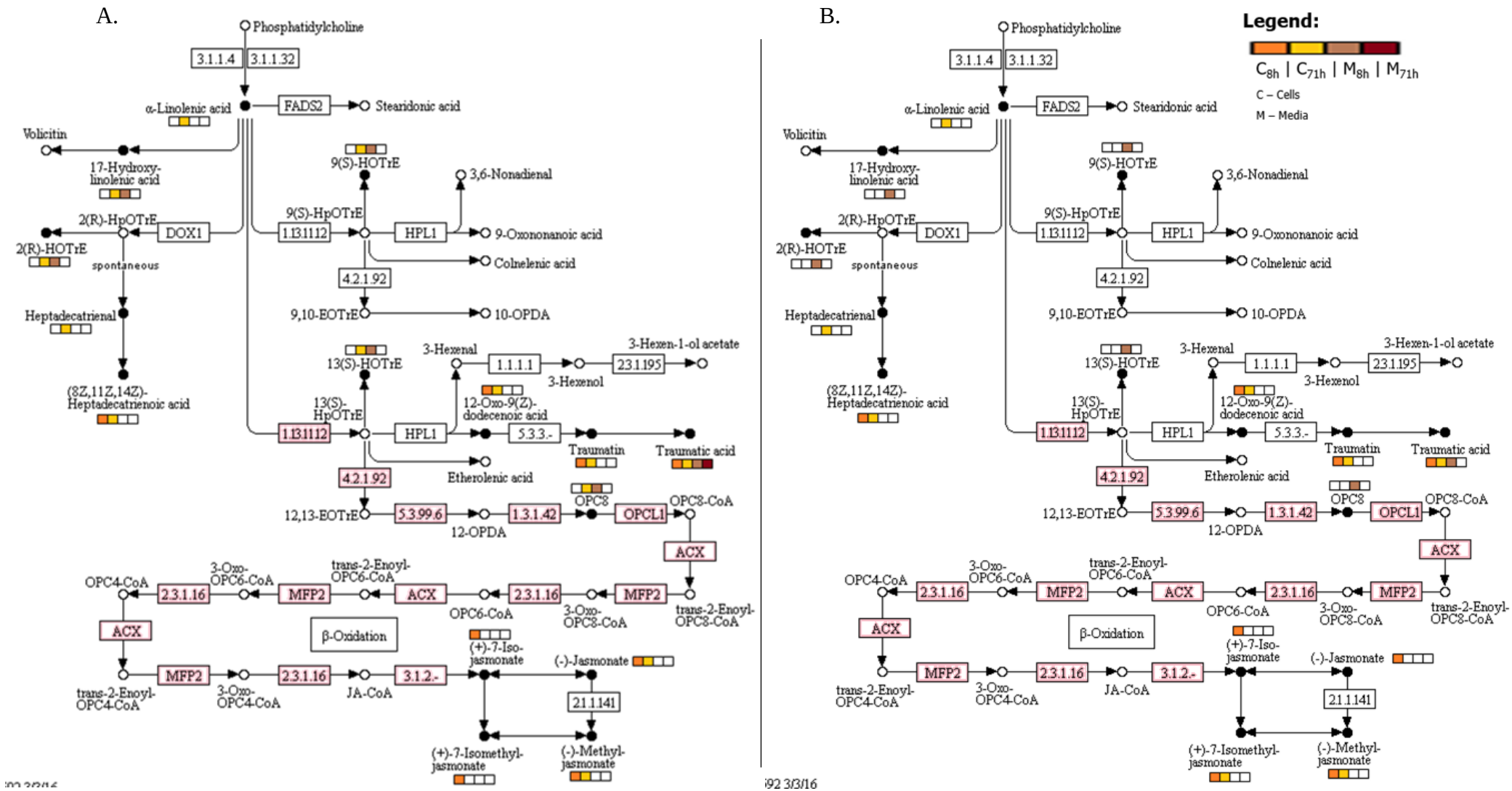


Figure 12. Alpha-linolenic acid metabolism in *Lachancea thermotolerans* in two different sugar conditions. A: corresponds to S1N2 growth; B: corresponds to S2N2 growth. Putatively identified compounds are marked as black full dots. Each square corresponds to a different sample, cells (C) and medium (S) at timepoints T2 (8h) and T9 (71h), (legend included in figure). Image adapted from KEGG.

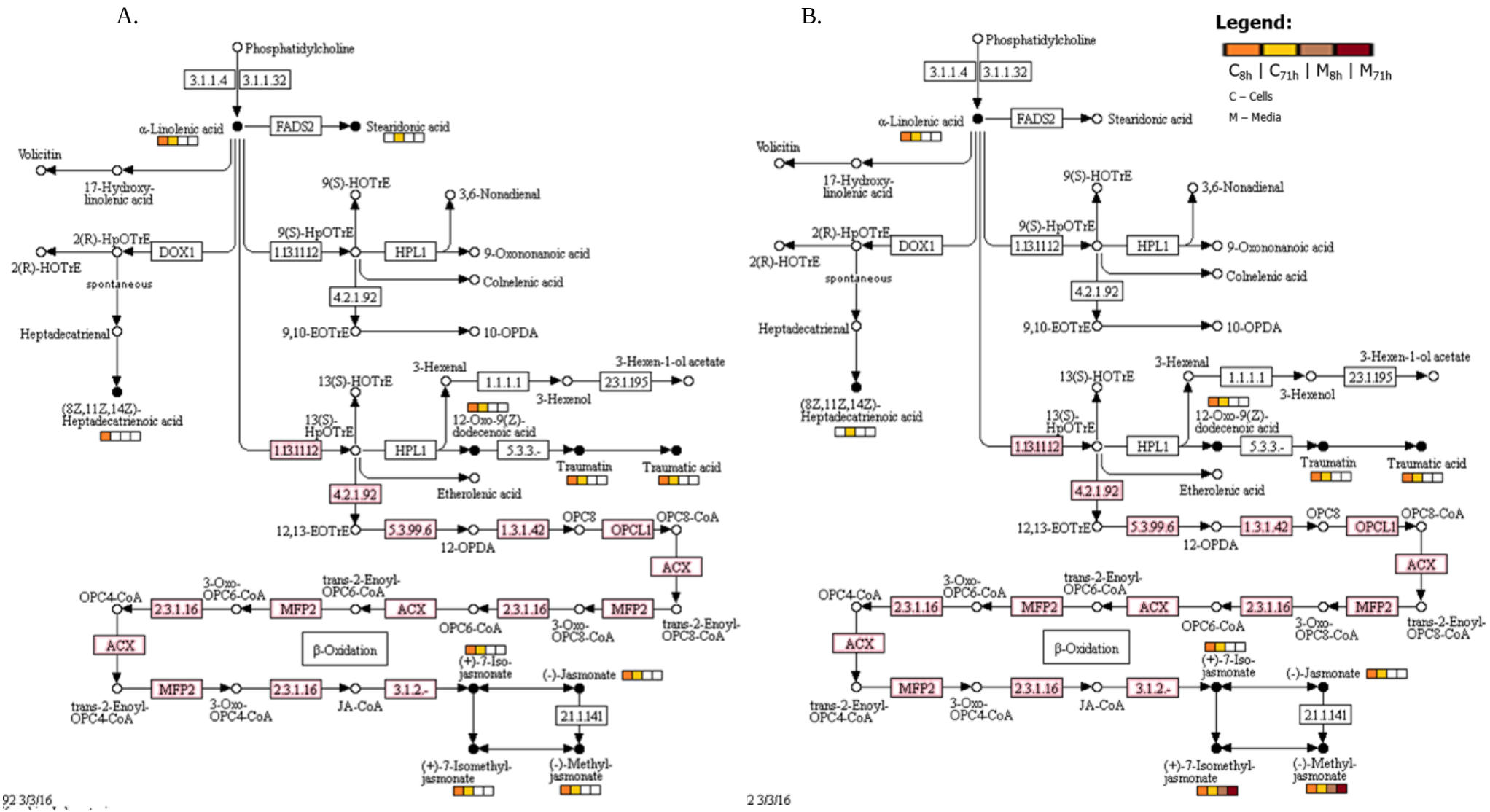


Figure 13. Alpha-linolenic acid metabolism in *Metschnikowia pulcherrima* in two different sugar conditions. A: corresponds to S1N1 growth; B: corresponds to S2N1 growth. Putatively identified compounds are marked as black full dots. Each square corresponds to a different sample, cells (C) and medium (S) at timepoints T2 (8h) and T9 (71h), (legend included in figure). Image adapted from KEGG.

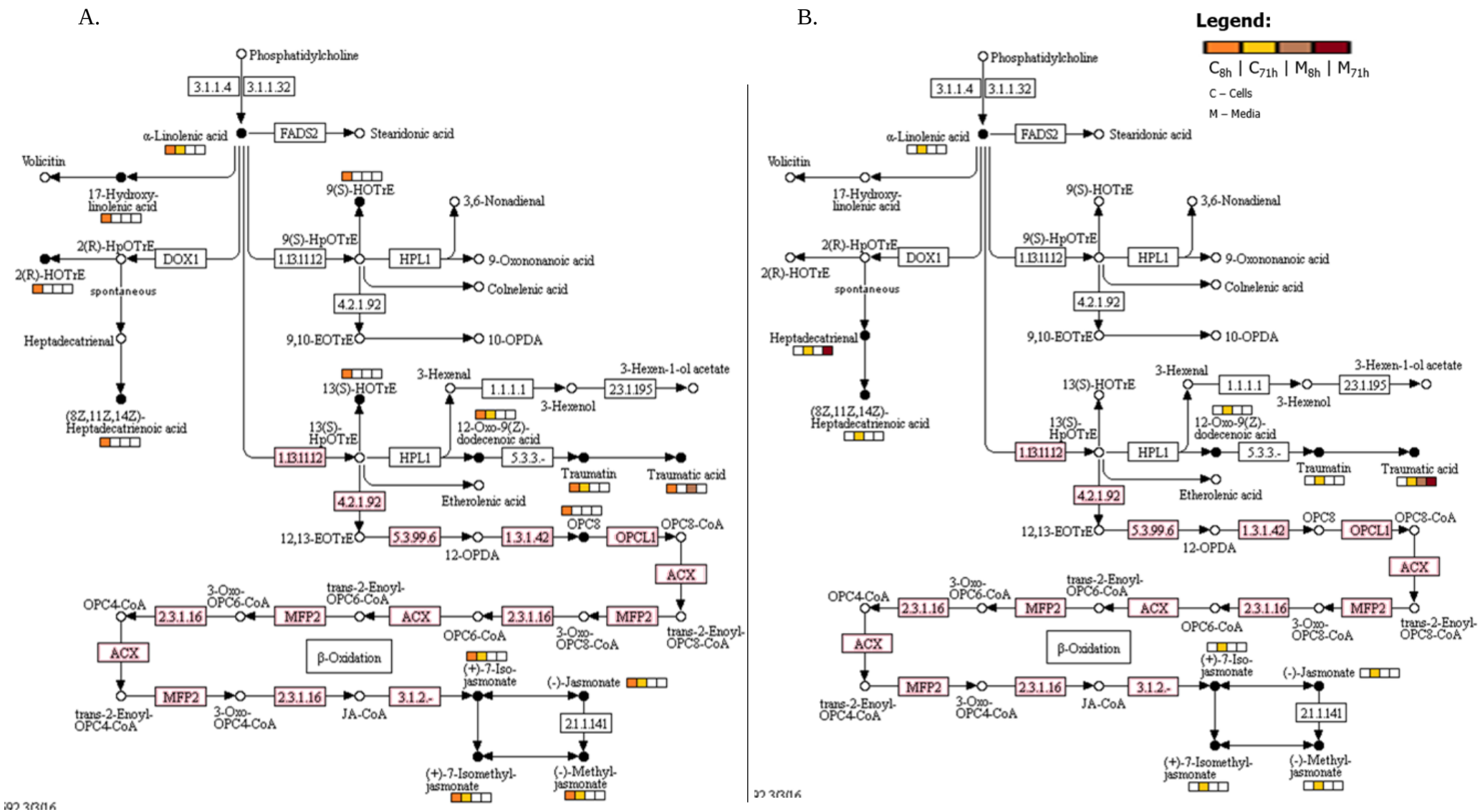


Figure 14. Alpha-linolenic acid metabolism in *Metschnikowia pulcherrima* in two different sugar conditions. A: corresponds to S1N2 growth; B: corresponds to S2N2 growth. Putatively identified compounds are marked as black full dots. Each square corresponds to a different sample, cells (C) and medium (S) at timepoints T2 (8h) and T9 (71h), (legend included in figure). Image adapted from KEGG.

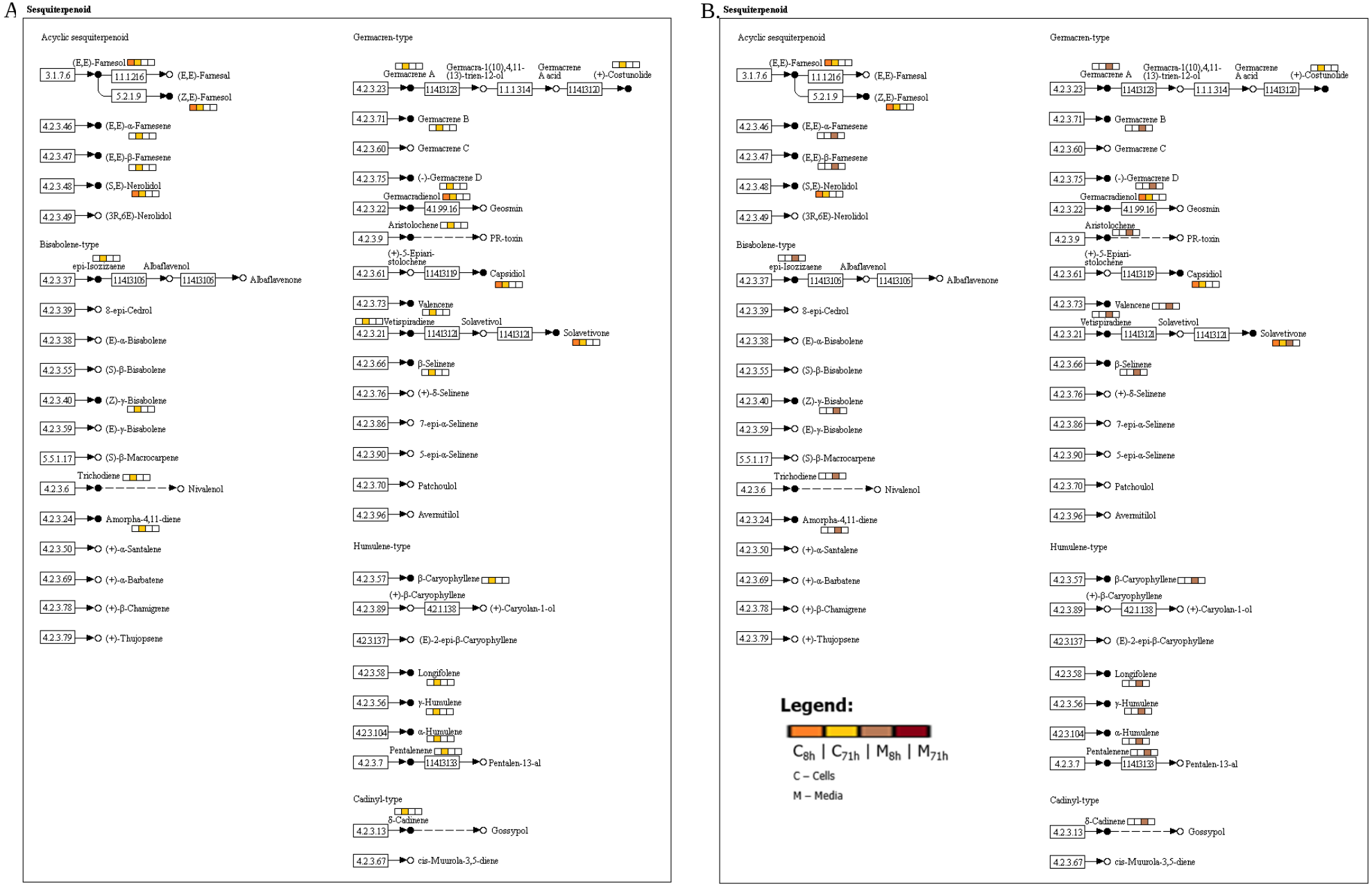
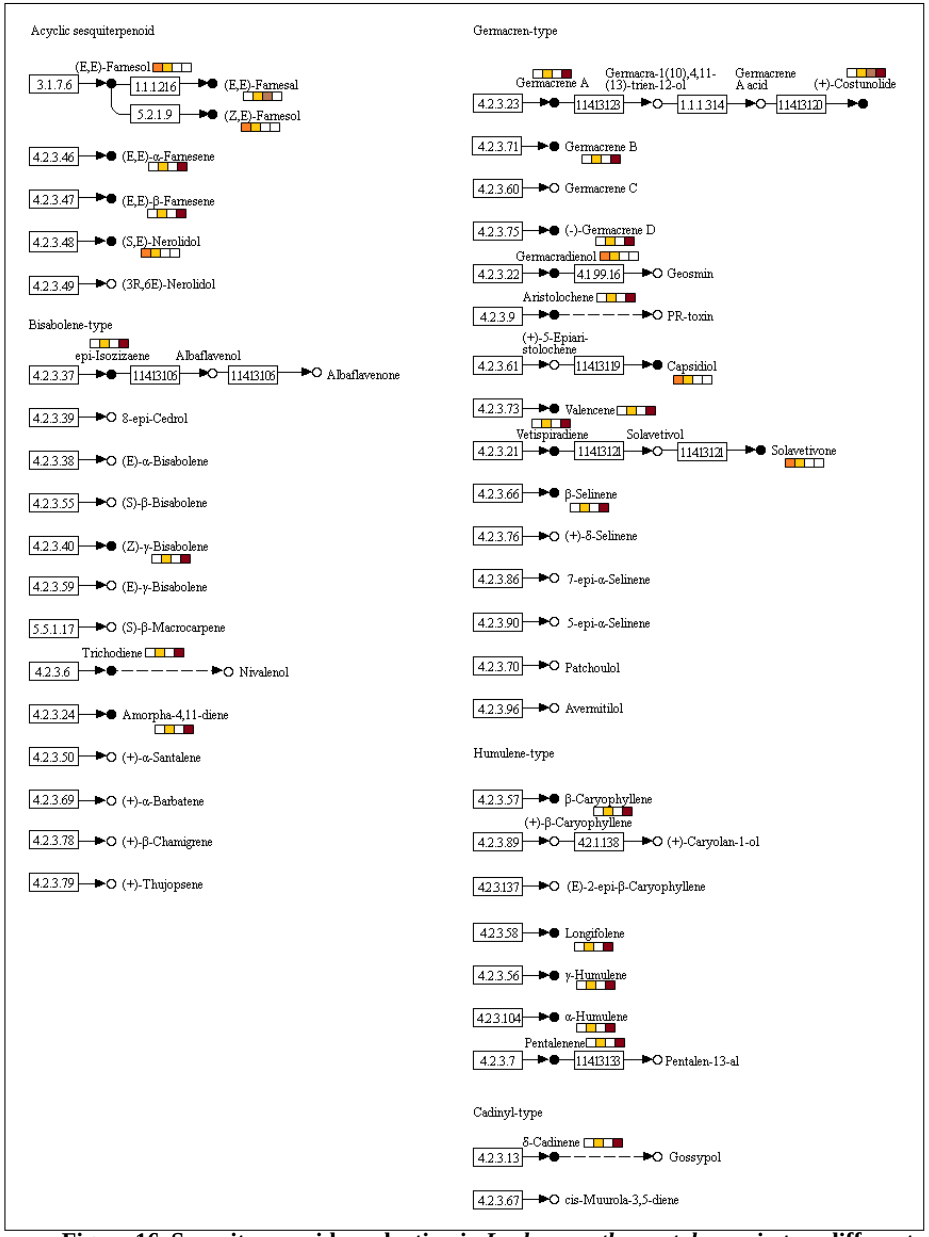


Figure 15. Sesquiterpenoid production in *Lachancea thermotolerans* in two different sugar conditions. A: corresponds to S1N1 growth; B: corresponds to S2N1 growth. Putatively identified compounds are marked as black full dots. Each square corresponds to a different sample, cells (C) and medium (S) at timepoints T2 (8h) and T9 (71h), (legend included in figure). Image adapted from KEGG.

A.



B.

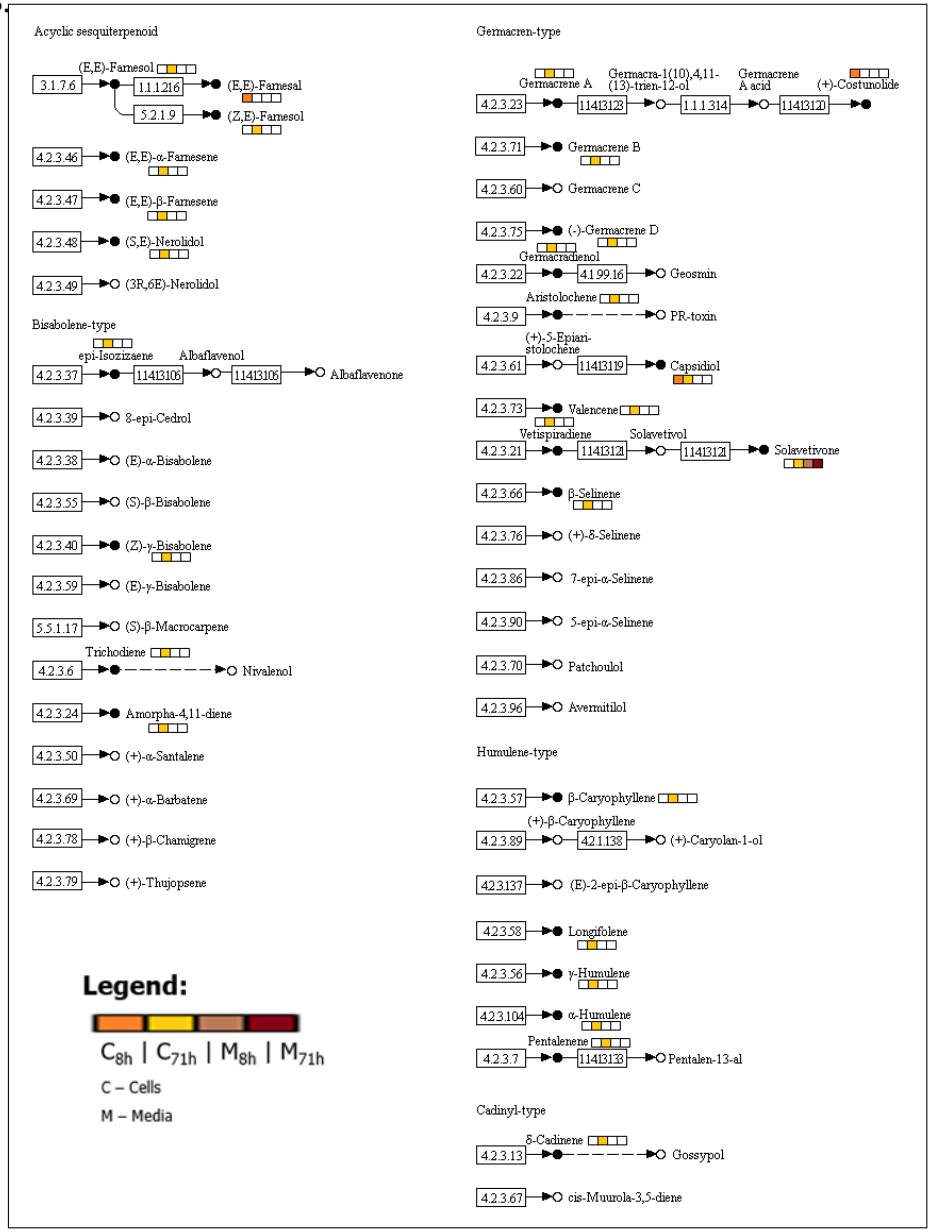
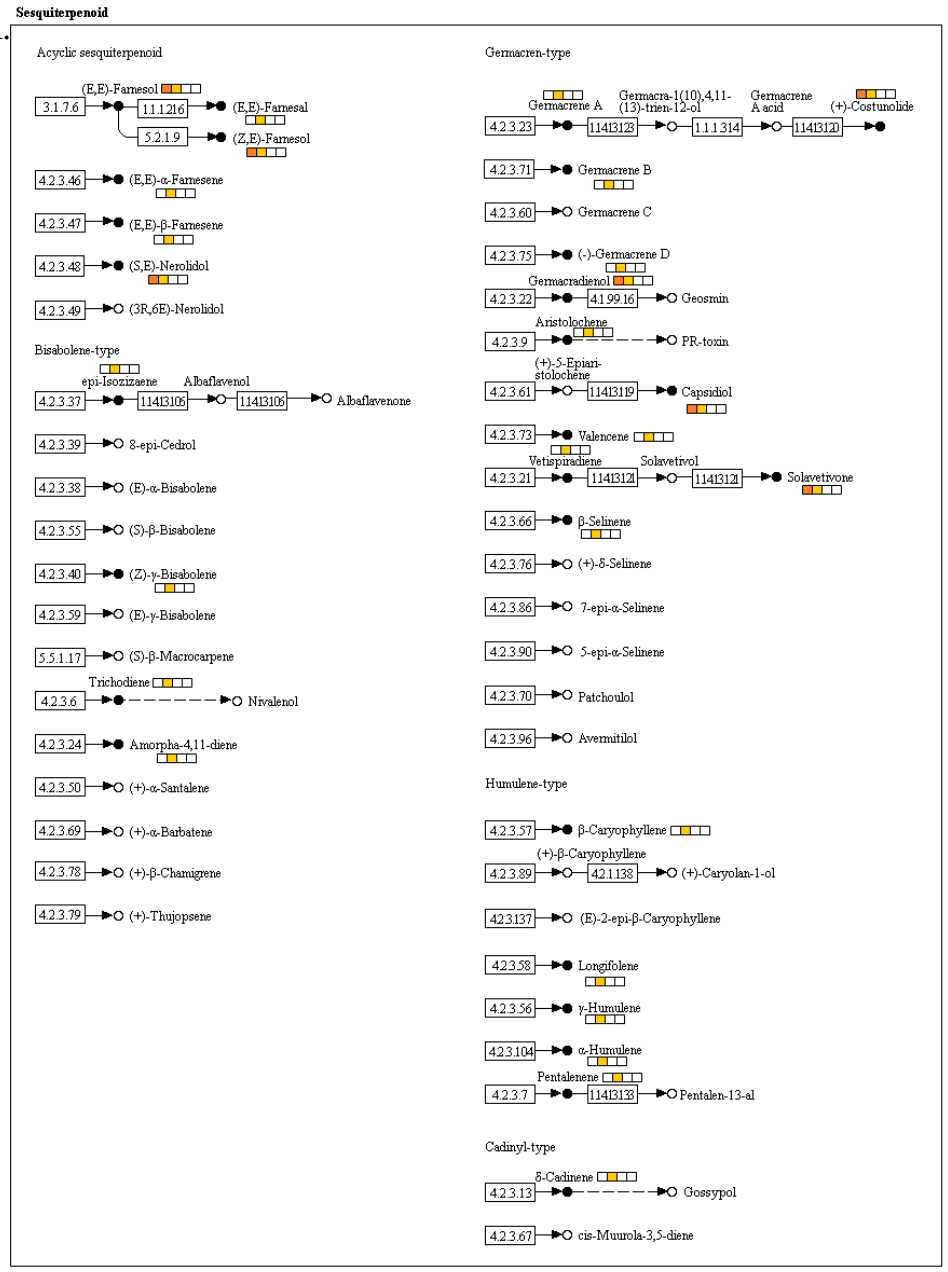


Figure 16. Sesquiterpenoid production in *Lachancea thermotolerans* in two different sugar conditions. A: corresponds to S1N2 growth; B: corresponds to S2N2 growth. Putatively identified compounds are marked as black full dots. Each square corresponds to a different sample, cells (C) and medium (S) at timepoints T2 (8h) and T9 (71h), (legend included in figure). Image adapted from KEGG.

A.



B.

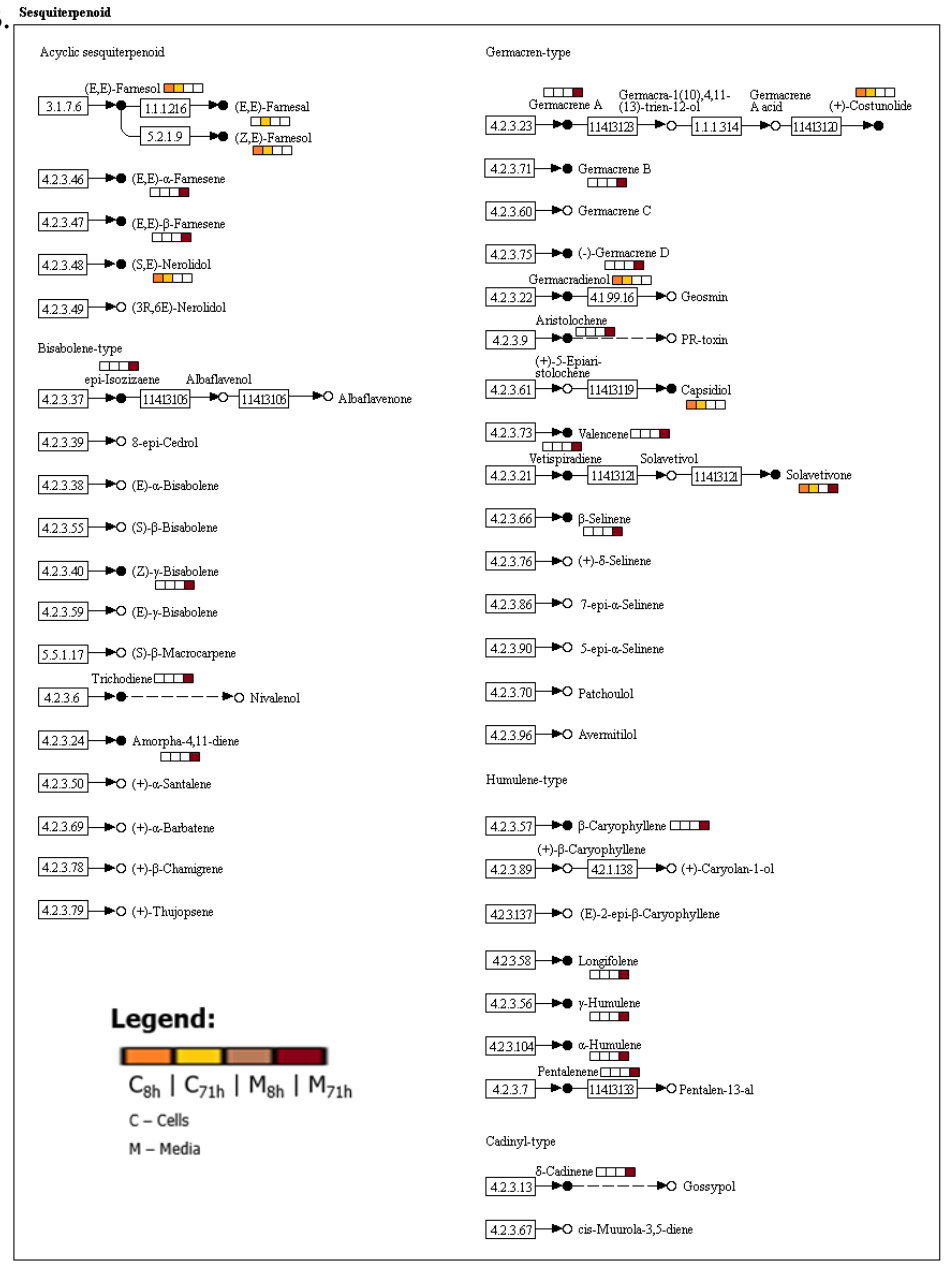


Figure 17. Sesquiterpenoid production in *Metschnikowia pulcherrima* in two different sugar conditions. A: corresponds to S1N1 growth; B: corresponds to S2N1 growth. Putatively identified compounds are marked as black full dots. Each square corresponds to a different sample, cells (C) and medium (S) at timepoints T2 (8h) and T9 (71h), (legend included in figure). Image adapted from KEGG.

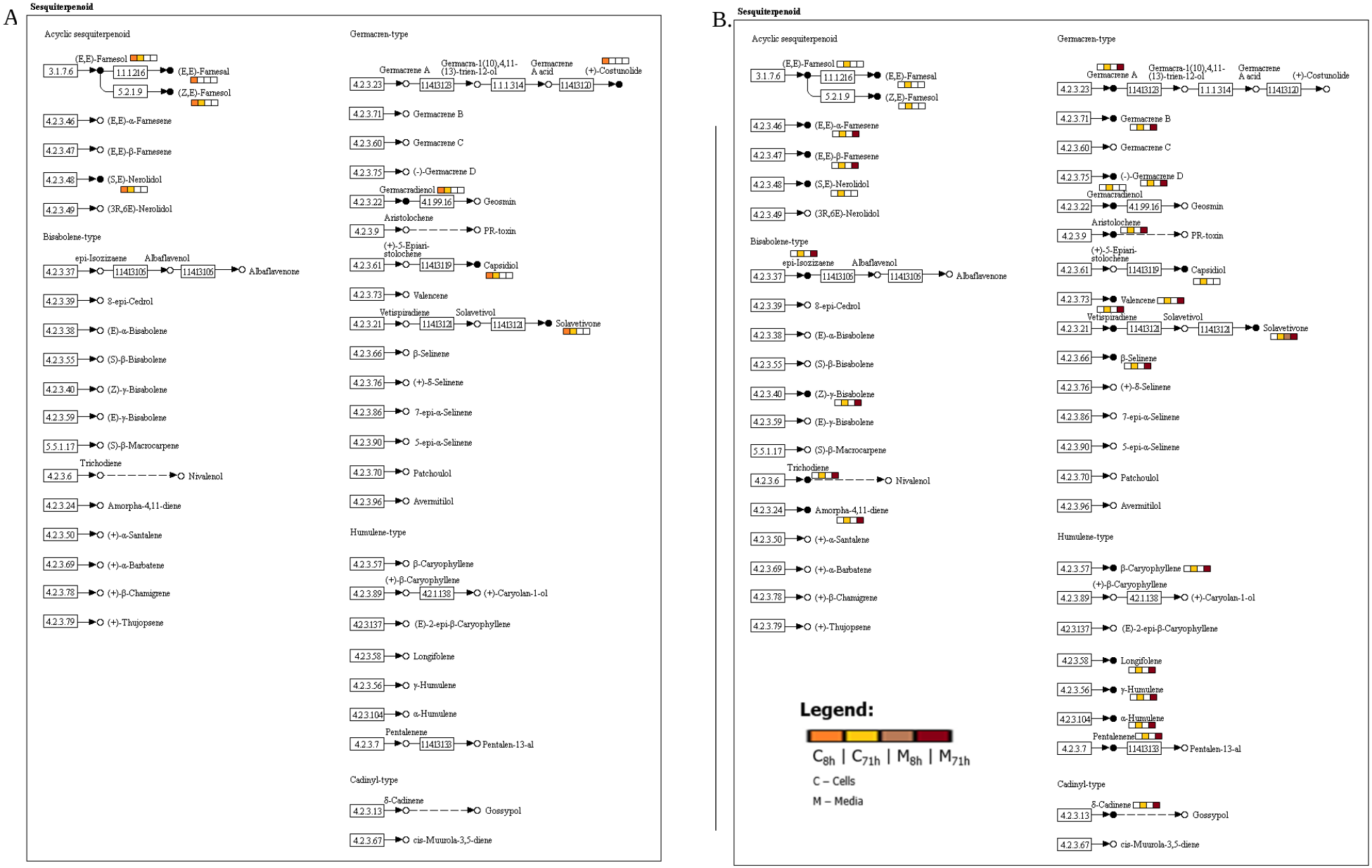


Figure 18. Sesquiterpenoid production in *Metschnikowia pulcherrima* in two different sugar conditions. A: corresponds to S1N2 growth; B: corresponds to S2N2 growth. Putatively identified compounds are marked as black full dots. Each square corresponds to a different sample, cells (C) and medium (S) at timepoints T2 (8h) and T9 (71h), (legend included in figure). Image adapted from KEGG.

Chapter 4. CONCLUSION

The growth analysis and the metabolic characterization of two selected non-*Saccharomyces* yeast species, *Lachancea thermotolerans* and *Metschnikowia pulcherrima*, was performed. Growth was followed in synthetic must in the presence of two concentrations of glucose and YAN, with the objective of assessing metabolic differences. Metabolite profiling of both cells and culture media revealed important differences in several metabolic pathways, particularly in linolenic acid metabolism and sesquiterpenoid synthesis.

Ripe grapes have an elevated sugar content with the main sugars being glucose and fructose, which have been increasing over time due to climate changes. The preferred carbon source for yeasts is usually glucose, factor attributed to characteristics of hexose transporters. Being glucose a central nutrient, it regulates many physiological changes within yeast cells, all beginning at the metabolic level (Heinisch et al., 2009; Otterstedt et al., 2004). As for nitrogen metabolism in yeast, it strongly influences the fermentation process. It is known that low nitrogen levels are linked to sluggish and stuck fermentations (Henschke & Jiranek, 1993). Nitrogen demand by fermenting yeasts can be influenced by fermentation conditions, namely sugar concentration. Literature describes an increased assimilation of nitrogen in response to presence of increased sugar concentrations attributing the phenomenon to a likely consequence of an increased need to maintain carbon catabolism due to extended fermentation (Graham H. Fleet, 2002; Henschke & Jiranek, 1993). Per biomass formed, the amount of nitrogen assimilated increased in response to an increase in sugar concentration in a way that the cellular nitrogen to carbon ratio increased. Studies suggest that higher nitrogen amounts may also be necessary for maintenance of metabolic functions when cells are under stressful conditions (Graham H. Fleet, 2002). It was expected that higher sugar concentrations in growth media would result in higher and faster biomass production when coupled to a higher nitrogen concentration, in a proportionate way such that the lower the sugar to nitrogen ratio the bigger will be the growth measured.

Results of *Lachancea thermotolerans* growth in the different media show an increase in growth rates when yeast cells are grown in higher glucose media (S2 conditions), independently of the nitrogen concentration (N1 or N2). To note that the higher growth rate happens in S2N2 medium, which suggests a higher fermentative metabolic capacity of *L. thermotolerans* yeasts for high sugar levels. Curiously, a higher number of organoleptically active compounds, are produced in S1N2 medium.

As for *M. pulcherrima*, the same is not observed. A slight increase of growth rate happens when N2 factor is present in S1 sugar conditions (S1N1 vs. S1N2), but the growth rate does not benefit with the same change of YAN when sugar concentrations are higher (S2N1 vs. S2N2). Since there is a great difference in growth variation percentage in S2 conditions when different N conditions are given, there might be metabolic differences that justify the apparent preference of *M. pulcherrima* yeast strain for lower YAN when sugar concentration is higher. *M. pulcherrima* yeasts produce a richer panoply of organoleptic compounds, especially in S2N1 and S2N2 media (in which the cells present the highest growth rates).

This was the first metabolic profiling analysis of *Lachancea thermotolerans* and *Metschnikowia pulcherrima*, grown in synthetic conditions that simulate the increase of sugar levels in grape must. The huge amount of information obtained require a thorough analysis and the assignment of metabolic pathways to better understand the metabolism of these two non-conventional wine-yeasts and the benefits of using them to produce new wines with different flavours and aroma.

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ANNEXES

Table 5. Synthetic grape must (SM) media composition.

Component		Amount (/L)	Stock []	Amount (per 300 mL)
Carbon				
Glucose	$C_6H_{12}O_6$	100 g | 115 g		30 g | 34.5 g
Fructose	$C_6H_{12}O_6$	100 g | 115 g		30 g | 34.5 g
Salts and organic acids				
L-Malic acid	$C_4H_6O_5$	3 g	100x	3 mL
Potassium sodium tartrate	$KNaC_4H_4O_6$	2.5 g	100x	3 mL
Citric acid	$C_6H_8O_7$	1.5 g	100x	3 mL
Dipotassium phosphate	K_2HPO_4	1.14 g	100x	3 mL
Magnesium sulphate	$MgSO_4.7H_2O$	1.23 g	100x	3 mL
Calcium chloride	$CaCl_2.2H_2O$	0.44 g	100x	3 mL
Trace Minerals				
Manganese chloride	$MnCl_2.2H_2O$	198.2 µg	1000x	300 µL
Zinc chloride	$ZnCl_2$	135.5 µg	1000x	300 µL
Iron chloride	$FeCl_2$	32.0 µg	1000x	300 µL
Copper chloride	$CuCl_2$	13.6 µg	1000x	300 µL
Boric acid	H_3BO_3	5.7 µg	1000x	300 µL
Cobalt dinitrate	$Co(NO_3)_2.6H_2O$	29.1 µg	1000x	300 µL
Sodium molybdate	$NaMoO_4.2H_2O$	24.2 µg	1000x	300 µL
Potassium iodate	KIO_3	10.8 µg	1000x	300 µL
Vitamins				
Myo-inositol	$C_6H_{12}O_6$	100 mg	500x	600 µL
Pyridoxine HCl	$C_8H_{12}NO_3Cl$	2.0 mg	1000x	300 µL
Nicotinic acid	$C_6H_5NO_2$	2.0 mg	1000x	300 µL
Calcium pantothenate	$C_9H_{17}NO_5$	1.0 mg	1000x	300 µL
Thiamine HCl	$C_{12}H_{17}N_4OS.HCl$	0.5 mg	1000x	300 µL
ρ-Amino benzoic acid	$H_2NC_6H_4COOH$	0.2 mg	1000x	300 µL
Riboflavin	$C_{17}H_{20}N_4O_6$	0.2 mg	1000x	300 µL
Biotin	$C_{10}H_{16}N_2O_3S$	0.125 mg	1000x	300 µL
Folic Acid	$C_{19}H_{19}N_7O_6$	0.2 mg	20x	15 mL
Nitrogen				
Diammonium phosphate (DAP)	$(NH_4)_2HPO_4$	0.660 g | 1.414 g		0.198 g | 0.4242 g

Table 6. Linear equations of growth in every used SM condition for *Lachancea thermotolerans* strain.

483|LCH	**YAN condition**		**Growth rate range**
Sugar condition	**N1 (140 mg/L)**	**N2 (300 mg/L)**	
S1 (200 g/L total sugars)	Y=0.0406x+6.2091	Y=0.0391x+6.2174	0.0015
S2 (230 g/L total sugars)	Y=0.0476x+5.9825	Y=0.0492x+6.0035	0.0016
Growth rate range	0.007	0.0101	

Table 7. Linear equations of growth in every used SM condition for *Metschnikowia pulcherrima* strain.

134|MET	**YAN condition**		**Growth rate range**
Sugar condition	**N1 (140 mg/L)**	**N2 (300 mg/L)**	
S1 (200 g/L total sugars)	Y=0.0213x+6.6186	Y=0.0224x+6.5509	0.0031
S2 (230 g/L total sugars)	Y=0.0257x+6.4275	Y=0.0231x+6.5215	0.0026
Growth rate range	0.0044	0.007	

Table 8. Growth rates comparison through percentages.

		Growth Media			
Strain		**S1N1**	**S1N2**	**S2N1**	**S2N2**
483|LCH	Growth rate equivalent percentage	100%	96.31%	117.24%	121.18%
	Growth rate variation percentage	-	Δ↓3.69%	Δ↑17.24%	Δ↑21.18%
134|MET	Growth rate equivalent percentage	100%	105.16%	120.66%	108.45%
	Growth rate variation percentage	-	Δ↑5.16%	Δ↑20.66%	Δ↑8.45%

Table 9. Statistical supplement for the two-way ANOVA analysis performed with 95% of confidence interval for *Lachancea thermotolerans* strain growth using NAUC's.

483|LCH				
Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significance**
Interaction	3.364	0.0049	**	Very significant
Time	90.30	<0.0001	****	Extremely significant
Column factor	0.7736	0.3005	Ns	Not significant
Subjects (matching)	1.428	0.0045	**	Very significant

Table 10. Statistical supplement for the two-way ANOVA analysis performed with 95% of confidence interval for *Metschnikowia pulcherrima* strain growth using NAUC's.

134|MET				
Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significance**
Interaction	1.237	0.1707	Ns	Not significant
Time	94.97	<0.0001	****	Extremely significant
Column factor	0.3140	0.5098	Ns	Not significant
Subjects (matching)	0.9983	0.0014	**	Very significant

Table 11. Identification of all samples shown in PCA analysis figures.

Sample number	Growth Media	Strain	Fraction	Timepoint
1	S1N1	CONTROL	-	-
2		483|LCH	S	T2
3				T9
4			C	T2
5				T9
6		134|MET	S	T2
7				T9
8			C	T2
9				T9
10	S1N2	CONTROL	-	-
11		483|LCH	S	T2
12				T9
13			C	T2
14				T9
15		134|MET	S	T2
16				T9
17			C	T2
18				T9
19	S2N1	CONTROL	-	-
20		483|LCH	S	T2
21				T9
22			C	T2
23				T9
24		134|MET	S	T2
25				T9
26			C	T2
27				T9
28	S2N2	CONTROL	-	-
29		483|LCH	S	T2
30				T9
31			C	T2
32				T9
33		134|MET	S	T2
34				T9
35			C	T2
36				T9

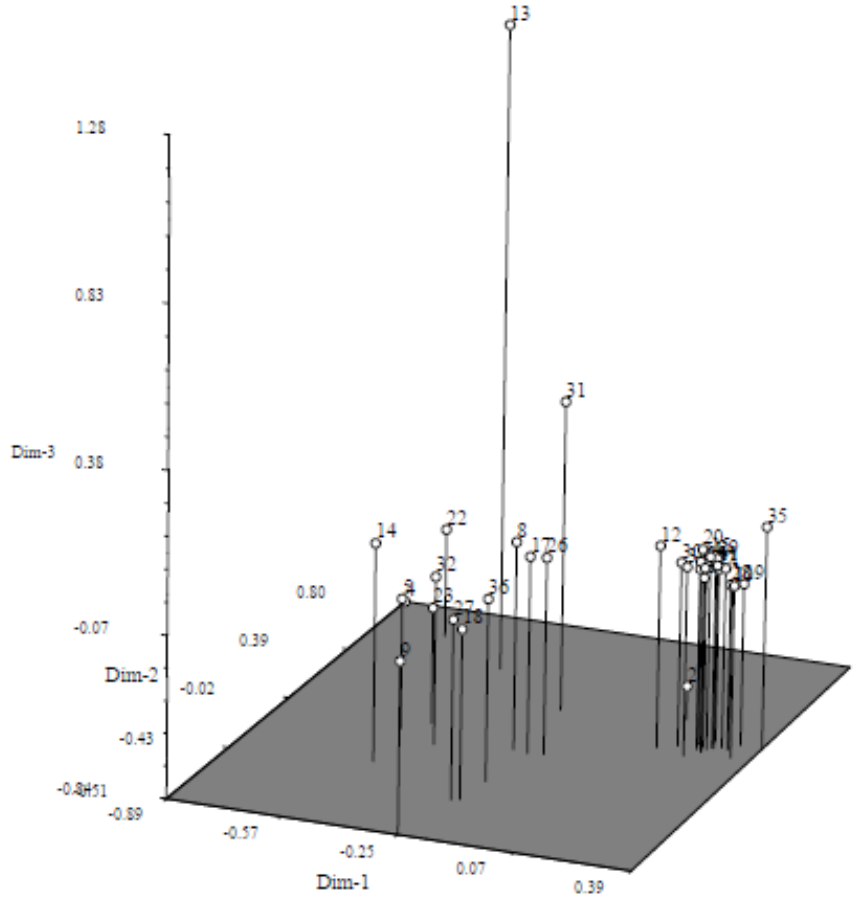

Figure 19. Resulting 3D projection of the first PCA performed, using the totality of samples data including non-inoculated media samples.

Table 12. List of compounds derived from metabolic analysis. Final list of relevant compounds resulting from the metabolic analysis showing chemical formulas and putative functions; * only the name of the first metabolite is shown when, for the same *m/z* value, several isomers can be putatively assigned.

Metabolite classification or function	Name of metabolite	Formula
Arachidonic acid derivative	Dihydro-nogalonic acid methyl ester	$C_{21}H_{18}O_8$
Lipid; fatty aldehyde	2-tridecene-4,7-diynal	$C_{13}H_{16}O$
Oxidative phosphorylation; secondary metabolites; or phthalates	Di-n-propylphthalate *	$C_{14}H_{18}O_4$
Lactone; or sesquiterpene; secondary metabolite; or toxin	Artecanin	$C_{15}H_{18}O_5$
Triglyceride prodrug of butyric acid; flavour	Tributyrin	$C_{15}H_{26}O_6$
Fatty acid choline; metabolite	Undecanoylcholine	$C_{16}H_{34}NO_2$
Flavonoid	7-Methoxy-2-methylisoflavone	$C_{17}H_{14}O_3$
Biosynthesis of antibiotics	Fortimicin A	$C_{17}H_{35}N_5O_6$
Prostaglandin; arachidonic acid metabolism	2,3-Dinor-8-iso-PGF2alpha*	$C_{18}H_{30}O_5$
Linoleic acid metabolism	Linolenic acid *	$C_{18}H_{32}O_4$
Ether lipid metabolism	LPA(P-16:0e/0:0)*	$C_{19}H_{39}O_6P$
Unsaturated fatty acids; fatty alcohols.	11,14-eicosadienoic acid*	$C_{20}H_{36}O_2$
Aldosterone antagonist; or anti-inflammatory flavour compound	Eplerenone*	$C_{24}H_{30}O_6$
Vitamin D3 derivative; alcohols	(20S)-3beta-Hydroxychol-5-en-24-oic Acid*	$C_{24}H_{38}O_3$
Indole alkaloid with antimitotic activity by inhibiting microtubule assembly	Vindoline	$C_{25}H_{32}N_2O_6$
Succinic acid derivative (volatile aroma compound)	(R)-3-Hydroxy-3-methyl-2-oxopentanoate*	$C_6H_{10}O_4$
Purine nucleotide biosynthesis	5-Aminoimidazole ribonucleotide*	$C_8H_{14}N_3O_7P$
Carboxylic acid	Azelaic acid*	$C_9H_{16}O_4$
Phenylpropanoid (2ndary metabolite) biosynthesis; or catecholamine metabolite (precursor to vanilactic acid)	5-Hydroxyferulate*	$C_{10}H_{10}O_5$
Bacterial origin; Yeast anticancer drug; or pentanoic acid; or essential oil component	1-Phenylpropyl acetate*	$C_{11}H_{14}O_2$
Tropane, pipiridine and pyridine alkaloid biosynthesis	(-)-Anaferine*	$C_{13}H_{24}N_2O$
Aromatic poliketides	4,4'-Dihydroxystilbene*	$C_{14}H_{12}O_2$
Structural derivative of eugenol and acetic acid; acetate ester; plant metabolite	1'-Acetoxyeugenol acetate	$C_{14}H_{16}O_5$
Iridoid monoterpenoid; anti-inflammatory	Monotropein	$C_{16}H_{22}O_{11}$
Organic heterocyclic compound	Karwinaphthol B	$C_{17}H_{20}O_4$
Hydroperoxy fatty acids	Methyl 10,12-dihydroperoxy-8E,13E,15Z-octadecatrienoate*	$C_{19}H_{32}O_6$

Glycerol precursor	DHAP(18:0e)	$C_{21}H_{43}O_6P$
Flavonoid	5-Hydroxy-7,8-dimethoxyflavanone 5-rhamnoside	$C_{23}H_{26}O_9$
Alkaloid biosynthesis; phytochemical	Demethylisoalangiside	$C_{24}H_{29}NO_{10}$
Vitamin D3 derivatives	(17E)-1alpha,25-dihydroxy-17,20-didehydro-21-norvitamin D3*	$C_{26}H_{40}O_3$
Flavonoid	Delphinidin 3-(2"-galloylgalactoside)	$C_{28}H_{25}O_{16}$
Vitamin D3 derivatives	(22E)-1alpha,25-dihydroxy-26,27-dimethyl-22,23-didehydrovitamin D3*	$C_{29}H_{46}O_3$
Diterpenoid	3'-N-Debenzoyl-2'-deoxytaxol;beta-Phenylalanoyl baccatin III	$C_{40}H_{47}NO_{12}$
Yeast metabolite; pentose interconversions	D-Ribitol 5-phosphate*	$C_5H_{13}O_8P$
Cyclic polyol; sweet	(-)-Quebrachitol*	$C_7H_{14}O_6$
Catecholamine or tyrosine metabolite; or phenolic compound found in red wine	3-(3,4-Dihydroxyphenyl)lactate*	$C_9H_{10}O_5$
Antibiotic or phenylpropanoid biosynthesis	1,2-Dihydroxy-3,4-epoxy-1,2,3,4-tetrahydronaphthalene*	$C_{10}H_{10}O_3$
Purine metabolism	Adenosine*	$C_{10}H_{13}N_5O_4$
Monoterpene	(+)-Iridodial*	$C_{10}H_{16}O_2$
Thiol	Glutathione	$C_{10}H_{17}N_3O_6S$
Aromatic poliketides	(E)-1-Methoxy-4-[2-(4-nitrophenyl)ethenyl]benzene*	$C_{15}H_{13}NO_3$
Aromatic poliketides; flavonoid	(2S)-Flavan-4-ol*	$C_{15}H_{14}O_2$
Aporphine alkaloid with anti-cancer, trypanosomal and anti-plasmodial activities; metabolite	Anonaine*	$C_{17}H_{15}NO_2$
Biosynthesis of type II poliketide products	Stealthin C	$C_{18}H_{13}NO_4$
Flavonoid	3',4',5-Trihydroxy-3,6,7-trimethoxyflavone*	$C_{18}H_{16}O_8$
Flavonoid	5,7,3'-Trimethoxy-4',5'-methylenedioxyflavone*	$C_{19}H_{16}O_7$
Alkaloid metabolite	Fagaridine*	$C_{20}H_{16}NO_4$
Flavonoidal alkaloid	(+)-Adlumine*	$C_{21}H_{21}NO_6$
Alkaloid biosynthesis	Lobelanine	$C_{22}H_{25}NO_2$
Monoacylglycerophosphoethanolamine	LysoPE(0:0/18:1(11Z))*	$C_{23}H_{46}NO_7P$
Monoacylglycerophosphoserine	PS(18:2(9Z,12Z)/0:0); 1-(9Z,12Z-octadecadienoyl)-glycero-3-phosphoserine	$C_{24}H_{44}NO_9P$
Hydroxy acid	3-Hydroxy-11Z-octadecenoylcarnitine*	$C_{25}H_{47}NO_5$
Glycerophospholipid metabolism	LysoPC(18:2(9Z,12Z))*	$C_{26}H_{50}NO_7P$
Diacylglycerol	DG(18:3(6Z,9Z,12Z)/20:5(5Z,8Z,11Z,14Z,17Z)/0:0)*	$C_{41}H_{64}O_5$
-	R replaced by H in L-Arginine ester	$C_6H_{13}N_4O_2R$
Amino acid	Arginine	$C_6H_{14}N_4O_2$
Sugar alcohol; sweet	D-Iditol*	$C_6H_{14}O_6$
Amino fatty acids	3-amino-octanoic acid*	$C_8H_{17}NO_2$

Biosynthesis of 2ndary metabolites; tryptophan metabolism	Indolylmethylthiohydroximate	$C_{10}H_{10}N_2OS$
Nucleotide analogue	2-Aminoadenosine	$C_{10}H_{14}N_6O_4$
Long chain alcohol related	(3R)-6-Hydroxy-3-isopropenyl-heptanoate*	$C_{10}H_{18}O_3$
Biosynthesis of antibiotics	3,4-Dihydro-7-methoxy-2-methylene-3-oxo-2H-1,4-benzoxazine-5-carboxylic acid	$C_{11}H_9NO_5$
Dioxin degradation	2,2',3-Trihydroxybiphenyl	$C_{12}H_{10}O_3$
Dicarboxylic acid	3-hydroxy-dodecanedioic acid	$C_{12}H_{22}O_5$
Monoacyl glycerols	R replaced by H in CDP-acylglycerol	$C_{13}H_{20}N_3O_{14}P_2R$
Glucoside of tyrolol; tyrosine metabolism; plants	Salidrosid	$C_{14}H_{20}O_7$
Sesquiterpene; isoprenoid	(+)-Hirusten-12-oic acid*	$C_{15}H_{20}O_5$
Pentose interconversions; glucoronate pathway	Epinephrine glucuronide	$C_{15}H_{21}NO_9$
Peptide hormone	Thyrotropin-releasing factor	$C_{16}H_{22}N_6O_4$
Monoterpene or glucid	10-Deoxygeniposidic acid*	$C_{16}H_{22}O_9$
Organic compound pyrethroid	Tefluthrin	$C_{17}H_{14}ClF_7O_2$
Diacylglycerophosphoethanolamine	PE(6:0/6:0)	$C_{17}H_{34}NO_8P$
Flavone; lipid	4-Ethyl-7-hydroxy-3-(p-methoxyphenyl)coumarin*	$C_{18}H_{16}O_4$
Fatty acid	R replaced by H in 9-Hydroxy-3,5,7,11,13,15,17-octaoxo-nonadecanoyl-[acp]	$C_{19}H_{23}O_9SR$
Sterol lipid	5alpha-androstane	$C_{19}H_{32}$
Fungal metabolite	Sulfamisterin	$C_{19}H_{36}NNaO_8S$
Phytochemical toxin	Gelsemicine	$C_{20}H_{26}N_2O_4$
Biosynthesis of antibiotics (macrolides)	Oleandolide	$C_{20}H_{34}O_7$
Monoacylglycerophosphoglycerol	PG(14:1(9Z)/0:0)	$C_{20}H_{39}O_9P$
Flavonoid biosynthesis	(2S)-5,6,7-Trihydroxyflavanone 7-glucoside*	$C_{21}H_{22}O_{10}$
Corticosteroid hormone	6alpha,9-Difluoro-11beta-hydroxypregn-4-ene-3,20-dione	$C_{21}H_{28}F_2O_3$
Steroid hormone	12alpha-Bromo-11beta-hydroxypregn-4-ene-3,20-dione	$C_{21}H_{29}BrO_3$
Tetrtahydronicotinamide adenine dinucleotide phosphate; R00129	(6S)-6-Hydroxy-1,4,5,6-tetrahydronicotinamide-adenine dinucleotide	$C_{21}H_{31}N_7O_{15}P_2$
N-glycan biosynthesis	Dolichyl b-D-glucosyl phosphate	$C_{21}H_{39}O_9P$
Phytochemical	(+)-Syringaresinol*	$C_{22}H_{26}O_8$
Arachidonic acid derivative	PGD2-EA*	$C_{22}H_{37}NO_5$
Flavonoid	Epigallocatechin 3-O-caffeate	$C_{24}H_{20}O_{10}$
Sulfite formation related	Benzyl viologen	$C_{24}H_{22}N_2$
Bile alcohols	(22R)-3alpha,7alpha,22-Trihydroxy-5beta-cholan-24-oic Acid*	$C_{24}H_{40}O_5$
Neuroprotective agent< potassium channel blocker	Linopirdine	$C_{26}H_{21}N_3O$

Plant secondary metabolite	Lithospermic acid	$C_{27}H_{22}O_{12}$
Flavonoid	6-Hydroxycyanidin 3-rutinoside*	$C_{27}H_{31}O_{16}$
Amino sugar metabolism	UDP-3-(3R-hydroxy-tetradecanoyl)-alphaD-glucosamine	$C_{29}H_{51}N_3O_{18}P_2$
Porphyrin and chlorophyll metabolism	Mesobilirubinogen	$C_{33}H_{44}N_4O_6$
Flavonoid glycoside	Licorice glycoside E	$C_{35}H_{35}NO_{14}$
Fraction of kinetensin; anti-inflammatory	Kinetensin 4-8	$C_{35}H_{47}N_{10}O_7$
	Putreanine or L-carnitinamide	$C_7H_{17}N_2O_2$
Antiseptic	4-Chloro-3,5-dimethylphenol	C_8H_9ClO
Folate biosynthesis	1-hydroxy-2-Oxopropyl tetrahydropterin*	$C_9H_{13}N_5O_3$